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(54) Title: TISSUE-SPECIFIC POPLAR PROMOTERS

(57) Abstract

The invention concerns the isolation and characterization of DNA sequences representing a caffeoyl-CoA-O-methyltransferase (CCoAOMT) promoter having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel or vessels. The cells adjacent to the vessel are xylem ray cells whereas the vessel can be a differentiating vessel.

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Tissue-specific poplar promoters

The present invention relates to isolated DNA sequences representing a *CCoAOMT* promoter or a functional part thereof having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel.

Background description to the invention

After cellulose, lignin is the major structural component of secondary thickened plant cell walls. It plays a very important role in vascular plants; it provides rigidity to the cell wall and hydrophobicity to the water-conducting vascular elements. Moreover lignin is often deposited after pathogen attack to prevent the spread of a pathogen. Lignin is a complex polymer of hydroxylated and methoxylated monolignol units that are termed *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). These units differ in their degree of methoxylation: the H unit is not methoxylated at the C5 position, the G unit once, and the S unit twice (see Figure 1, lignin biosynthesis pathway). These units are subsequently dehydrogenated by peroxidase(s) and possibly laccase(s) to form mesomeric radicals which polymerise to form the complex lignin network (Boudet et al., 1995). Gymnosperm lignin consists mainly of G units. Angiosperm dicot lignin incorporates both G and S units whereas grass lignin consists of all three units. The S/G ratio impacts the physical properties of lignin. Lignin content increases with maturity in stems (Jung and Vogel, 1986), and the composition changes with advanced maturity towards a progressively higher S/G ratio (Buxton and Russell, 1988). In the paper industry, it has been long a problem to remove lignin from cellulose by chemical paper pulping, because of the huge cost and the large environmental pollution (Tien, 1987). Both a high lignin concentration (Albrecht et al., 1987) and a low S/G ratio have a negative impact on lignin extractibility during chemical pulping. Lignin also limits forage digestibility. Even a small decrease in lignin content has a significant positive impact on paper pulping and digestibility and

therefore profitability (Casler, 1987). Therefore, there is considerable interest in the potential for genetic manipulation of the lignin level and/ or composition to improve digestibility of forages and pulping properties of trees (Dixon et al., 1994).

The biosynthesis pathway of lignin precursors proceeds through the common phenylpropanoid pathway starting from phenylalanine and leading to the synthesis of cinnamoyl-CoAs. Subsequently, the cinnamoyl-CoA esters are channelled into the lignin branch pathway to produce cinnamyl alcohols. The methylation of the 3- and / or 5-hydroxyl group of hydroxycinnamic acids is an important step influencing lignin composition because the chemical structures of the monomeric lignin precursors differ only in 3- and / or 5-methoxyl groups on the aromic ring. The *O*-methyltransferases (OMTs) involved in lignin formation have been characterised in a number of species (Grisebach, 1981; Bugos et al., 1991; Gowri et al., 1991; Collazo et al., 1992; Davin and Lewis,1992). Caffeic acid *O*-methyltransferase (COMT. EC2.1.1.68) has long been considered as the sole methylation pathway involved in lignification (Neish, 1968; Grisebach, 1981 ; Lewis and Yamamoto, 1990). However, there is now increasing evidence suggesting that the *O*-methylation of the lignin precursors may also occur at the level of the hydroxycinnamoyl-CoA esters. A specific *O*-methytransferase involved in the methylation of caffeoyl-CoA (caffeoyl-Coenzyme A 3-*O*-methytransferase;CCoAOMT) was initially characterised in parsley cell suspensions (Kneusel et al. , 1989) and in carrot cell suspensions (Kuhnl et al., 1989). In parsley cell suspension cultures, the addition of a fungal elicitor induced both *p*-coumaroyl-CoA 3-hydroxylase (CCoA-3H) and CCoAOMT activities. The resulting feruloyl-CoA however, first was thought to be incorporated uniquely into cell wall-bound feruloylated polymers implicated in the defence response. However, Ye et al (1994) and Ye and Varner (1995) recently suggested that the CCoAOMT mediated pathway is dominant in lignifing *Zinnia* tracheary elements (TES) *in vitro,* ; they showed that CCoAOMT, but not COMT is induced during tracheary element formation. Ye and Varner (1995) suggested that, like in White birch and *Arabidopsis*, tracheary elements may be composed of mainly G units and that therefore COMT might not be expressed under these conditions. Nevertheless, although in these published works CCoAOMT was suggested to be involved in lignification, a direct link has still to be found. Furthermore, it was found that in COMT down-regulated tobacco (Atanassova et al,1995) and poplar (Van Doorsselaere et al, 1995) the S/G ratio was

decreased. The modification of the lignin composition in these transgenic plants was mainly due to a decrease in the number of S units and an increase in the level of G units. These results demonstrate that the activity of COMT mainly controls the level of S units and therefore it was hypothesised that the production of G units in COMT down-regulated plants may result from the functioning of a CCoAOMT-mediated methylation pathway.

Detailed description of the invention.

The present invention concerns the isolation and characterisation of two genes encoding CCoAOMT in poplar which share high homology in the coding region but are diverse in their regulatory parts. To study its specific expression at the cellular level in plants, histochemical analysis have been performed in transgenic poplars harbouring chimeric p*CCoAOMT-GUS* constructs. In addition, the CCoAOMT protein has been immunolocalised. The data show that both genes are differentially expressed in phloem fibers. In the xylem of the stem, both genes are expressed preferentially in the ray cells next to vessel elements for which it is known that the lignin is mainly composed of G units. These data show that CCoAOMT is involved in lignification for providing G units to the cell wall, even after autolysis of the cytoplasm.
The invention further relates to an isolated DNA sequence according to figure 1 B (also called SEQ ID NO 1) which represents a *CCoAOMT* promoter or a functional part thereof having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel.
Furthermore an isolated DNA sequence according to figure 1 C (also called SEQ ID NO 2) which represents a *CCoAOMT* promoter or a functional part thereof having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel belongs to the current invention.
The cells adjacent to the vessel as defined can be xylem ray cells and xylem fibers whereas in addition the vessel can be a differentiating vessel.
Part of the invention is a promoter sequence conferring expression in living vessel elements and/or in ray cells, adjacent to living or autolysed vessel elements and/or in fibers adjacent to said living or autolysed vessel elements, with the proviso that said

expression does not occur constitutively in xylem fiber cells or ray cells that are not adjacent to said vessel elements.

In this last described situation the expression conferred by the promoter or a functional part thereof according to the invention occurs preferably in those ray cells comprising a so-called pith to the vessel as is clearly demonstrated in figures 5A and 5B respectively.

With "a functional part of a promoter" is meant in this description, any part or a fragment of a promoter that can still induce gene expression in the cells as disclosed herein. Such a fragment or part can be used alone or in combination with a second promoter and/or parts or fragments of said second promoter.

With "gene expression" is meant a sequence of events that results in the synthesis of RNA starting from DNA (so called transcription) regardless whether or not the resulting RNA is translated into a protein.

Furthermore, the expression of both above mentioned chimeric genes was markedly induced by wounding and fungal infection. GUS analysis showed that the expression of both chimeric genes was only induced when lignin was deposited at the wounded zone. The similar correlation between the induced expression of chimeric genes and the induced formation of lignin was detected at the site of fungal infection as well. So it has been shown that the regulation of both chimeric *CCoAOMT* genes is involved in lignification not only during normal development but also in response to wounding and pathogen infection.

To the scope of the invention also belongs a recombinant DNA comprising any of the isolated DNA sequences according to the invention whereas an embodiment of the current invention is represented by a plant or tree cell comprising said recombinant DNA integrated in its genome. Furthermore to the invention belongs a transgenic plant or tree comprising said cell and its progeny thereof but also seed, seedlings, roots and the like.

In order to localize promoter regions potentially involved in the transcriptional control of the *CCoAOMT* gene, several deletions were generated by removing part of the 5' flanking sequences of both *CCoAOMT* genes (Fig. 14), and examined their effect on gene expression in transgenic poplars. 4-13 individual transformants per construct were analyzed.

For the deletions of the *PtCCoAOMT1* gene, differences were found in the tissue specific pattern of GUS expression between plants containing the full-length promoter (*pBINPOP1*) and the various promoter deletions. As indicated in Table 3 GUS activity was undetectable in poplar transformed with the *pBIN1DD1* (-114) promoter-GUS construct. In poplar transformant with the deletion *pBIN1DC2* (–184) no GUS activity could be detected in the xylem tissue, whereas faint GUS activity was detected in phloem fibres, cortex and periderm (Fig. 15). The fact that the *pBIN1DC2* (–184) is sufficient for directing expression in bark tissue, a *cis*-positive regulatory element (domain +B) could lie between –184 and –114 bp upstream of the translation start site (Fig. 17). Construct of *pBIN1DB3* (-199) which is 15 bp longer than *pBIN1DC2* failed to direct expression in any tissue, suggesting that this additional 15 nucleotides (which includes an AC-II element) contains a *cis*-negative regulatory element (domain -B) for controlling the expression in bark tissue. In the transformants containing *pBIN1DA5* (-456), GUS staining remained in the xylem vessels and xylem ray cells adjacent to these vessels and in the periderm as for the full-length promoter *pBINPOP1*. However, it was found that *pBIN1DA5* (-456) lost expression in companion cells and conferred additional expression in cambial ray initial cells (Fig.16). This observation suggested the presence of two domains between –456 and –199 bp upstream of the translation start site of the *PtCCoAOMT1* promoter: one domain (+CR) positively controls expression in cambial ray cells, the other domain (V) positively regulates expression in vessels and adjacent ray cells. Furthermore, the comparison between the expression patterns conferred by the full-length promoter *pBINPOP1* and *pBIN1DA5* suggests the presence of a domain (CC) that directs expression in companion cells and a domain (-CR) that negatively controls the expression of *CCoAOMT* in cambial ray cells between –1993 and –456 bp upstream of the translation start site of the *PtCCoAOMT1* promoter.

For the *PtCCoAOMT2* promoter, the deletion of *pBIN2DD1* (–110) and *pBIN2DB3* (–195) resulted in a complete loss of expression. *PBIN2DA4* (–497) however directed expression as the full-length *PtCCoAOMT2* promoter. The –497 fragment of *PtCCoAOMT2* promoter is sufficient to control the cell-specific expression pattern, suggests that *cis*-acting positive regulatory elements, which control the expression in xylem vessels and in adjacent ray cells (domain V) and in phloem fibres (domain F), are located between nucleotides –497 and –195.

The transgenic poplars containing the promoter deletions were also analyzed upon wounding. The GUS staining revealed that *pBIN1DA5* and *pBIN2DA4* were significantly expressed at the wounding zone of leaves 3-5 days post wounding as their full-length promoters. The P-HCl staining indicated that lignin was deposited at the site of wounding as well. The other deletions did not shown any GUS activity at the wounding zone, suggesting that a *cis*-acting positive regulatory element (domain W) for response to wounding is located between –456 and –199 of the *PtCCoAOMT1* promoter and between –497 and –195 of the *PtCCoAOMT2* promoter.

Taken together, the two full-length *CCoAOMT* promoters and their deletions revealed several domains of importance for regulated expression, as summarized in Table 3. The putative regions that could contain cis-acting element(s) for cell- and tissue- specific expression are summarized in Figure 17. By nucleotide sequence comparison of *CCoAOMT* promoters from different species several conserved regions were identified between the different sequences of each promoter and between the both promoters (Fig. 13). These regions are probably cis-regulatory elements for controlling the cell- and tissue-specific expression of CCoAOMT.

The invention is further explained by non-limiting examples given hereunder.

EXAMPLES

1. Genomic clones and copy number of *CCoAOMT* genes

Genomic clones were isolated from a poplar genomic library (*P. trichocarpa* cv Trichobel), by using a cDNA encoding CCoAOMT from poplar (*P. trichocarpa* cv Trichobel) as a probe. Six individual positive plaques were screened, and two of them, which are designated as *Pop1* and *Pop2*, respectively, were studied in detail (Fig.1 A-D). Comparison of the nucleotide sequences of these two genes, 3.8kb and 3kb in length respectively, to the *CCoAOMT* cDNA shows that both genes consist of five exons and four introns with identical intron locations. Both deduced polypeptides have 340 aa, with a calculated MW of 27.8 kDa for *Pop1*, and 27.9 kDa for *Pop2*. Alignment of the two genes revealed that they share high identity in the coding regions (93.9% at amino acid level), but are significantly divergent in the noncoding parts. In *Pop1*, a putative polyadenylation signal AATAAA is found 82 nucleotides

downstream from the TGA stop codon whereas in *Pop2,* an ATAGTT sequence is located at 68 nucleotides downstream of the stop codon. The transcription start site was determined by comparison with the corresponding cDNA sequences. The TATA motif of *Pop1* and *Pop2* was found at position 125 bp and 121 bp upstream from the translation start site ATG , respectively. According to the *CCoAOMT* cDNA sequence, the transcription start site is situated at 71 bp upstream the translational start point in *Pop1* and 69 bp in *Pop2*. Several conserved regions were found in the promoters of these two genes.

2. Expression patterns conferred by the *CCoAOMT* promoters

In order to study the tissue and cell specific expression of both genes, the two promoters of *Pop1* and *Pop2,* 2kb and 1.3 kb in length, respectively, were fused to the *ß-glucuronidase* (GUS) reporter gene (Figure 2). Poplar (*P. tremula x P. alba*) was stably transformed with both chimeric constructs via *Agrobacterium tumefaciens*-mediated transformation. Histochemical GUS assays were done on three-month-old greenhouse-grown transgenic poplars.

Figure 3 shows a transversal section through a young internode, stained with phloroglucinol-HCl for lignin, and analysed for GUS activity. The primary xylem stains red. At this stage GUS activity, driven by the two promoters according to the invention, was detected in the xylem ray cells between the vessels, in the pith parenchyma cells surrounding the inner primary xylem vessels, and within developing vessels themselves (Fig. 4). No GUS staining can be seen in the lignified parts of primary xylem, between the vessel bundles (Fig. 3 and 4).

In older internodes, GUS activity conferred by the two promoters is present in the secondary xylem shown in a cross section through the stem (Fig. 5). Observations under higher magnification of the xylem tissue clearly demonstrated that GUS activity was preferentially localised in xylem ray cells just next to vessels and within young developing vessels themselves (Fig. 5 and 6). Most expression was detected in the younger parts of the xylem, less towards the inner part of the xylem. No GUS activity was detected in xylem fibers throughout the whole stem, except for fibers that were adjacent to vessels (see further).

Although both promoters gave a very similar expression pattern in the xylem, they were differentially regulated in phloem tissue. *Pop1* conferred expression

specifically in companion cells of the phloem and in the cells surrounding the phloem fibers, whereas *Pop2* conferred expression within the phloem fiber cells (Fig. 7 and 8).

A similar cell specific expression pattern was detected in petioles and leaves (Fig. 9 and 10); GUS analyses revealed that both chimeric genes had similar expression patterns in the xylem of these organs, but had a different expression pattern in the phloem. *Pop2* conferred expression preferentially in phloem fibers (Fig. 9B and 10B) while *Pop1* preferentially in phloem parenchyma cells (Fig. 9A and 10A).

Figures 11A and 11B show that both chimeric genes were similarly expressed in xylem ray cells next to vessels and in phloem fibers in roots.

In addition, both chimeric genes were strongly expressed in the phellogen and phelloderm of the periderm. The blue staining of the GUS activity was localised in the cell layer adjacent to the cell layer that stained red with phloroglucinol-HCl (Figure 7 and 8). GUS activity was also detected in the meristem (figure 11C).

3. Response to mechanical wounding

The biosynthesis of lignin is not only an essential process under normal conditions but is also believed to be crucial in responses to stress such as mechanical wounding (Matern and Grimmig, 1994; Nicholson and Hammerschmidt, 1992).

To study the involvement of *CCoAOMT* in response to mechanical wounding during the formation of a lignin barrier, the spatio-temporal expression of the two chimeric genes was histochemically characterised in leaf tissue at different periods after wounding. Histochemical assays in combination with GUS staining and phloroglucoinol-HCl staining revealed that no GUS activity nor lignified cells were detected in leaf samples harvested one, two, three and four days after wounding. When the necrophylactic layer became visible surrounding the cutting site in leaf samples five days after wounding, phloroglucinol-HCl staining revealed a barrier, suggesting the deposition of lignin or lignin-like material. At this stage, GUS staining showed that expression conferred by both *Pop1* and *Pop2* promoters was associated with the lignified barrier. When the barrier was well developed in leaf samples harvested seven days after wounding, no GUS activity was detected anymore.

Table 2 summarises the expression characteristics conferred by both promoters.

4. Expr ssion of both gen s is induced by fungal infection

To investigate whether expression of *CCoAOMT* is a response to pathogen infection, detached leaves from transgenic and wild type poplar were sprayed with spores of the fungus *Melampsora pinitorca*, a natural pathogen for poplars belongs to *Leuce* section. Three days after infection, *in vitro* leaves, induced GUS activity was observed. Subsequently the infected leaves die within 5 to 10 days without appearing uredosores. In contrast, in the leaves of green house grown plants seven days after infection, the orange uredosores became visible and necrotic lesions developed at the site of infection. At this stage, GUS activity driven by the both promoters was strongly induced in the cells surrounding these uredosores and necrotic lesions, whereas no GUS staining was observed before the formation of uredosers. The blue colour of the staining is closely associated to the lignified region at the infection sites, indicating a correlation between the induced expression of *CCoAOMT* chimeric genes and lignification in necrotic lesions both *in vitro* and *in vivo* leaves.

5. Immunocytochemical localisation of CCoAOMT

In order to confirm the GUS analysis, immunolocalisations were performed using stem sections in which secondary growth was apparent, using polyclonal antibodies for CCoAOMT. Immuno-fluorescence microscopy revealed labelled proteins as a fluorescent green colour. CCoAOMT was localised intensively within differentiating vessels, and specifically in xylem ray cells just next to lignifying vessels (Figure 12B). In addition, labelling was detected on the secondary thickened walls of the phloem fibers (Figure 12C). No immunolabeling was observed in xylem fibers, except for the fibers that were adjacent to vessel elements. In these fiber cells, immunolabel was detected preferentially at that site of the cell that was adjacent to a vessel. The results indicate that the pattern of localisation of CCoAOMT in stem tissue was very similar to the pattern obtained by histochemical GUS assays. Hardly any signal could be observed in sections of the same stem treated with rabbit pre-immune serum (Figure 12A)

6. Cloning and sequencing the promoters from different species of poplar

It was reasoned that the *cis*-elements important for tissue specific expression should be conserved between a series of allelic forms of the promoter itself. In order to identify possible elements responsible for the tissue specific expression pattern, a

PCR-based promoter amplification strategy was developed. By alignment of the two *CCoAOMT* promoters, conserved sequences were identified. These conserved sequences were used to design primers that would allow the amplification of the homologous sequences from other poplar species (Figure 1D). The PCR products were cloned into a pGEM-T vector and sequenced. By comparing these sequences with each other, several conserved sequences, present in all promoters were identified (Fig. 13). The AC-element II, a putative regulatory element involved in UV light and /or elicitor regulation in promoters of other plant phenylpropanoid biosynthesis genes such as PAL and 4CL (Table 1), was conserved in both promoters.

For sake of clarity in order to better understand the invention and the materials used therein a "Material and Method" section is given hereunder.

Material and methods

1. Screening of a genomic library

A lambda FIX II custom genomic library (Stratagene, USA) from poplar (*Populus trichocarpa* cv Trichobel) was screened by using a ^{32}P-labelled poplar *CCoAOMT* cDNA as a probe. Six positive clones were isolated. Lambda DNA was prepared and digested by various restriction enzymes, and the fragments homologous to the cDNA probe were subcloned into a pBluescript vector (Stratagene).

2. DNA sequence analysis

The nucleotide sequence of genomic subclones in Bluescript and a series of overlapping deletions was determined by dideoxynucleotide sequencing.

3. The *Pop-GUS* fusion constructions

The promoter fragments (*Pop*1, 2kb and *Pop*2, 1.3kb) were translationally fused to the *uid A* coding region in plasmid pGUS1 (Peleman et al., 1989). The fragments containing the chimeric promoter-GUS fusions were cloned into the binary vector pBIN19 (Bevan, 1984).

4. Plant material and transformation

Poplar plants used in stable transformation experiments corresponded to the INRA clone 717-1B4 (*Populus tremula x Populus alba*). This line was selected because of its susceptibility to *Agrobacterium tumefaciens* infection (Leplé et al., 1992). The *popGUS* constructs in pBIN19 were mobilised to *Agrobacterium* strain C58C1Rif harbouring the plasmid pMP90 by the freeze-thaw method described by Zham et al. (1984). Poplar was transformed with the chimeric constructs following the approach described by Leplé et al. (1992). *In vitro* plants were maintained on half MS medium at 24 °C with a photo-period of 16 h light and 8 h darkness. About two-month old plantlets were transferred to a greenhouse. Transgenic poplars were grown in the greenhouse at 21 °C with the same light cycle. GUS analyses were performed after three months of growth in the greenhouse.

The following species of poplar were used to make promoter comparisons between different species of poplar: *P.trichcarpa, P.euphratica, P.laurifolia, P. nigra, P. deltoides, P.alba, P. maximowiczii, and P. ciliata* (see Figure 13).

5. Histochemical assays

Histochemical staining for GUS activity was performed according to Jefferson et al. (1987). Stems, roots, leaves and petioles from transgenic plants were sectioned with a vibroslicer (Laborimpex, Brussels, Belgium), fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer, pH 7.0, for 30 min at room temperature. The sections were kept in the same phosphate buffer. GUS staining was carried out by incubating sections with X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), K3Fe(CN)6, and K4Fe(CN)6, for 1-4 h. Staining was allowed to proceed at 37 °C until blue stain developed in the samples (1 to 4 h).

Lignin was revealed by staining with phloroglucinol-HCl according to Speer (1987).

6. Cloning and sequencing of *CCoAOMT* promoters from different poplar species

Based on a homology sequence alignment between the two *CCoAOMT* promoters, a 5' primer was chosen from a conserved sequence localised at about 900 bp upstream of the initiation codon and a 3' primer 20 bp downstream of the

initiation codon (Fig.1-D). Genomic DNA, that was prepared according to Shure et al. (1983), was used as template for PCR. PCR reactions were perfomed using 500 ng of genomic DNA and 10 pmol of each primer. The reaction mixture was first kept at 94 °C for 4 min followed by 30 cycles under following conditions: denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 50 seconds on a PCR machine. The PCR product was cloned directly into the pGEM-T vector (Promega).

7. Preparation of antibodies directed against CCoAOMT

CCoAOMT was expressed in *E.coli* as a fusion protein using the pGEX (Pharmacia Biotech) expression vector . The fusion protein was purified using the Glutathione Sepharose 4B Redipack columns (Pharmacia Biotech). Purified native fusion protein was used to immunise a rabbit following the standard protocol. After cutting the purified fusion protein with trombine protease, a second rabbit was immunised with a CCoAOMT sample cut out from SDS-PAGE. Western blotting procedures were standard. (Western blots of poplar xylem extracts using the antiserum raised against the fusion protein and against the CCoAOMT gel sample, showed predominantly two bands, +/- 29 kDa and a weaker +/-26 kDa. The upper band corresponded to CCoAOMT as evidenced by microsequencing the corresponding spots on a two-dimensional gel. The nature of the less intense hybridising band of lower molecular weight is still unknown).

8. Deletions (5'-upstream) in *CCoAOMT* promoter-GUS constructs

A series of *CCoAOMT* promoter fragments were created by PCR using a pair of specific primers which were homologous to either the 5' upstream or the coding region, as shown in Figure 1-B and 1-C. The PCR products were ligated into the *pGEM-T* vector by using the pGEM®-T Vector System I Kit (Promega) and subsequently translationally fused to the *uidA* gene in plasmid *pGUS1* (Peleman *et al.*, 1989) using the restriction sites *NcoI* and *SacI*. The chimeric GUS fusions were subsequently cloned as *XbaI* fragment into *pBIN19* (Bevan, 1984; Figure 14). All the plasmids were verified by restriction digestion and sequencing. Those with desired orientation were selected and transferred into *Agrobacterium tumefaciens* by the freeze-thaw method described by Zham *et al.* (1984).

9. Lignin staining

Lignin was revealed by phloroglucinol-HCl (P-HCl) staining according to Speer (1987). Sections and samples were incubated for 2 min in phloroglucinol solution (1% in ethanol/water 92/8 v/v), then mounted in 25% HCl. The Maüle reaction was used to distinguish lignin rich or poor in syringyl monomers (Monties 1987). For the Maüle reaction, samples were immersed for 5 min in 1% $KMnO_4$, and rinsed with H_2O. Subsequently, the samples were destained for 1 min in 25% HCl, washed with H_2O, and mounted in 32% of NH_4OH.

10. Wounding and pathogen infection

Mechanic wounding was performed on the petioles of three months old greenhouse plant by making half cm long vertical slit with a scalpel. Wounded petiole regions were excised at day 1, 2, 3, 4, 5, and two weeks post wounding. Two months old transgenic plants were grown in Weck-pot *in vitro* condition. A half leaf was cut off using a sterile scalpel, and a 2-mm width leaf tissue from cutting site was cut and stored directly at -70°C as control. The wounded plant was continuously incubated at the same *in vitro* condition. Subsequently, a 2-mm width of wounded leaf tissue from cutting site was corrected after wounding for 1, 2, 3, 4, 5 days, respectively. The harvested samples were then used for GUS assays.

For pathogen infection, the detached leaves were sprayed with spores of fungus (*Melampsora pinitorca*) on the under side, in a concentration of 200,000 spores/ml. The infected leaves were floated up side down on water in Petri dishes and were incubated at 22° C in a greenhouse for various periods.

Strictly for the case of clarity some key elements of the current invention are discussed below in more detail.

The tissue and cell specific expression pattern of CCoAOMT has been demonstrated in a woody plant. The GUS reporter system has permitted to reveal the temporal and spatial expression patterns conferred by the *Pop1* and *Pop2* promoters. Histochemical analysis of GUS activity showed that both *Pop1* and *Pop2* were

predominantly expressed in the vascular tissue of stems, leaves, petioles, and roots. The GUS data presented here are consistent with the tissue printing analysis described earlier by Ye and Varner (1995). The chimeric *Pop1-GUS* and *Pop2-GUS* constructs exhibited some different and some overlapping patterns of GUS expression, and these are summarised in Table 2. The tissue-specific expression of the *Pop1* and *Pop2* promoters were strictly correlated with tissues undergoing active lignification such as xylem and phloem fibres.

In the xylem of stems throughout the whole plant, both *Pop1* and *Pop2* promoters conferred expression in the xylem ray cells adjacent to vessels and in differentiating vessel themselves. Immunolocalisiation studies confirmed the accumulation of CCoAOMT in the ray cells next to vessels and within differentiating vessels themselves. A strong preferential expression is conferred by the promoters in ray cells adjacent to vessel elements. For many of the lignin biosynthesis genes, the tissue specific expression has been analysed, either by RNA tissue printing, or by promoter-GUS fusions. In bean expression conferred by the *PAL*-promoter was also localised preferentially in xylem ray cells (Bevan et al. 1989). Hauffe et al. (1991) reported the localisation of GUS activity under control of the *4CL* promoter preferentially in xylem at the onset of tracheary element differentiation, and strictly in xylem ray cells positioned between highly lignified tracheary elements (vessels and fibres). Smith et al. (1994) provided further evidence by complementary immunolocalisation, that PAL and 4CL accumulate in cells adjacent to the metaxylem in bean hypocotyls. However, PAL and 4CL are also involved in the synthesis of other phenylpropanoid end products. The role of xylem parenchyma cells in lignin synthesis is still questionable. Recently, Feuillet et al.(1995) and Hawkins (1997) reported that the promoter of *CAD* in *Eucalyptus* was expressed in regions undergoing active lignification i.e. phloem fibres, differentiating xylem, ray parenchyma cells, and vascular cambium. The *CAD* promoter was active throughout the ray, thus next to vessels but also fibers.

It is indicated according to the invention that *CCoAOMT* promoter activity, unlike those of *PAL, 4CL* and *CAD* which are constitutively present in xylem ray cells, is strictly located in cells just next to vessels. It is strongly suggested that CCoAOMT is closely correlated to vessel lignification.

It has been shown by Osakabe et al., (1996) that lignin in the cell wall of vessel elements is predominantly G lignin. This is in contrast to the lignin in fibers cell walls, which consists of both G and S units in comparable amounts. Also in white birch and *Arabidopsis*, lignin in vessel cell walls incorporates predominantly G units (Saka and Goring, 1988; Chapple et al., 1992). A high level of CCoAOMT in ray parenchyma cells adjacent to living as well as dead vessels has been shown. These data therefore suggest that CCoAOMT is indeed involved in the synthesis of coniferyl alcohol units. In addition, the studies suggest that in living cells, the coniferyl alcohol is synthesised within the vessel and the adjacent living ray cells provide coniferyl alcohol to the adjacent vessel when it is dead. Monolignols are likely also provided by the adjacent xylem fiber cell; by immunolocalisation it was shown that CCoAOMT protein was present at the site of the cell that was adjacent to the vessel. The specific activity of the promoter in or adjacent the vessel can be used for several applications. It opens the possibility to reinforce vessel elements in transgenic plants that have altered fiber characteristics, allowing e.g. the construction of plants with fibers with a modified lignin content, without affecting the function of the vessels of the plant.
Apart from lignin related products, the promoter system may be used to produce products, other than monolignols, that can be transported to the vessel. As a non limiting example, the promoter may be used to produce small antifungal peptides in the cells adjacent to the vessel. These peptides may be transported into the vessel and from there, to the whole plant, to confer resistance against fungal attack.

It has also been shown that both *Pop1* and *Pop2* promoters conferred expression in the periderm. The strong promoter activity in the periderm was associated with the formation of lignified cells in the phellem. Such observation suggests that CCoAOMT plays an important role in the generation of a protective dermal layer in plants. In addition, since the GUS activity was detected in the cell layer adjacent to the lignified layer, this supports the hypothesis that monolignols can be provided by adjacent cells.

In phloem, the promoter of *Pop2* confers expression within the phloem fiber cells, whereas the promoter of *Pop1* confers expression in the cells surrounding the phloem fibers. In xylem, however, both promoters are expressed very similarly. This suggests that *cis* elements necessary for xylem expression might be different from those needed for phloem expression. Hence, it might be possible to identify and

isolate elements that are needed specifically for expression associated with vessel elements. In the 4CL promoter, a negative *cis*-acting element which represses phloem expression has been identified. This element appears to be responsible for restricting vascular expression to the xylem (Hauffe et al., 1993).

Alignment of the two promoter sequences allowed us to identify putative elements involved in cell- and tissue-specific expression of these two genes. The AC-II element (or box L) present in the promoters of *Pop1* and *Pop2* . had initially been identified by *in vivo* footprint experiments as light and elicitor-responsive promoter element in the parsley *PcPAL-1* and *Pc4CL* genes (Hauffe et al. 1993, Lois et al. 1989). They were later found to be present in various other genes of the phenylpropanoid pathway from other plants (Table 1). Da Costa e Silva (1993) isolated a DNA-binding factor BPF1 for the corresponding AC elements. They observed an induction of BPF1 synthesis in response to elicitor treatment in parsley cell suspension cultures. In spite of the convincing functional relevance, transient expression assays with promoter-reporter gene constructs in parsley were not sufficient to explain the responsiveness to light or elicitor, and a combinatorial interaction of these AC-elements even with exonic sequences has been suggested (Hauffe et al., 1993). The other homologous sequences, as identified in the consensus sequence (Fig. 13), or parts of it, optionally in combination with the AC-elements, may play a role in the determination of the tissue specificity of the promoter, especially in determining the expression in living vessel elements, in ray cells adjacent to vessels elements or in fibers adjacent to vessel elements.

Since the *CCoAOMT* promoters confer expression in cells adjacent to vessel elements but not next to fiber cells, it follows that specific signals must be transported from the vessel to the adjacent cells, even when the vessel element has undergone autolysis. In this respect, "cells, adjacent to vessel elements" are defined as those cells that can be reached by a signal molecule that is diffusing out of a vessel; cells that are not reached by the signal are considered as not adjacent. Said signal can also be a certain pressure originated from water that is transported through said vessel. Such a pressure can be captured by for instance a membrane protein whivh simply transports the signal concerned. For the expression of other lignification genes that are "constitutively" expressed in the living ray cells, such a signal would not a priori be necessary, because the expression of these genes could be part of the

default set of genes that are expressed in the ray cells. The presence of *cis* elements in the promoter of the *CCoAOMT* genes that interact with vessel derived factors can thus be anticipated. It is foreseen that these response elements are also located - at least partly - in the consensus sequence that is derived from the promoter comparison. These response elements, as part of the *CCoAOMT* promoter, or as separate elements in combination with another promotor sequence can be used to produce proteins in a reaction upon the signal coming from the vessel. As a non limiting example is mentioned that the signal is a toxic compound and the protein is a detoxifying enzyme, or the example where the signal is the result of a microbial contamination, and the protein an antimicrobial agent.

The close spatial association between promoter activity and lignified tissues strongly suggests the involvement of these two genes in the lignification process. Transgenic poplars in which the expression of CCoAOMT is reduced will unravel the precise role of CCoAOMT in lignification.

Table 1. Putative cis-acting elements and positions on various *PAL, 4CL* and *CCoAOMT* gene promoters

Promoter	Position	AC-element II	Reference
PvPAL2	-123	TCTCCACCAACCCC	Cramer et al. 1989
AtPAL1	-135	TCTCAACCAACTCC	Ohl et al. 1990
AtPAL2	-132	TCTCACCCACCCCT	Warner et al. 1995
PsPAL2	-204	TCTCAACCAACCAC	Yamada et al. 1994
PopPALg2b		TCTCAACCAACCCC	Osakabe et al. 1996
PopPALg1		TCTC ACCAACCAC	Osakabe et al. 1996
PcPAL1	-107	TCTC ACCTACCAA	Lois et al. 1989
PcPAL2	-107	TCTC ACCTACCAA	Logemann et al. 1995
PcPAL4	-268	TCTC ACCAACCCC	Logemann et al. 1995
Pc4Cl1	-128	TCTC ACCAACCCC	Logemann et al. 1995
Pc4Cl2	-128	TCTC ACCAACCCC	Logemann et al. 1995
St4Cl1	-17	TCTC ACCAACCAC	Joos et al. 1992
St4Cl2	-168	TCTC ACCAACCAC	Joos et al. 1992
PcCCoAOMT	-120	TCTC ACCAACCGC	Grimmig and Matern,1997
Pop1(CCoAOMT)	-205 *	CCTC ACCAACCCC	this invention
Pop2(CCoAOMT)	-201 *	CCTC ACCAACCCC	this invention
	-498 *	CCTC ACCAACCCC	this invention
Consensus		TCTC ACCAACCCC	
		C T AA	

At, Arabidopsis thaliana; Pc, Petroselinum crispum; St, Solanum tuberosum; Ps, Pisum sativum; Pv, Phaseolus vulgaris; Pop, Populus kitakamiensis (Yuriko Osakabe), and *Populus trichocarpa* (this work). Position numbers counted from the transcriptional start except the ones marked with * which were from the translation start codon.

Table 2. Promoter activity in transgenic poplar

	CCoAOMT1-GUS	CCoAOMT2-GUS
Stem :		
young internode	· xylem ray cells between vessels · parenchyma cells surrounding inner vessels	· xylem ray cells between vessels · parenchyma cells surrounding inner vessels
older internode	· xylem ray cells next to vessels * companion cells * parenchyma cells surrounding phloem fibres	· xylem ray cells next to vessels * phloem fibres
Leaf and petiole:	· xylem ray cells	· xylem ray cells * phloem fibres
Root:	· xylem ray cells next to vessels * phloem fibres	· xylem ray cells next to vessels * phloem fibres
Periderm:	· pellogen and phloderm	· pellogen and phloderm
Bud	. meristem	.meristem

Table 3. The deletions of *CcoAOMT* promoters activity in stems of transgenic poplar

	number of analysed primary transformants	primary tissue	secondary tissue	response to wounding
pBINPOP1 (-1993)	8(15)	primary xylem, base of hair	vessel, ray cells next to vessel, periderm	yes
		pith ray cell surrounding inner of xylem	companion cell	
pBIN1DA5 (-456)	5(6)	primary xylem, base of hair	vessel, ray cells next to vessel, periderm	yes
		pith ray cell surrounding inner of xylem	ray initial cells in cambial zone	
pBIN1DB3 (-199)	13(13)	undetected	undetected	no
pBIN1DC2 (-184)	10(10)	undetected	phloem fibres, cortex and periderm	no
pBIN1DD1 (-114)	8(8)	undetected	undetected	no
pBINPOP2 (-1362)	8(15)	primary xylem, base of hair	vessel, ray cells next to vessel, periderm	yes
		pith ray cell surrounding inner of xylem	phloem fibres	
pBIN2DA4 (-497)	4(7)	primary xylem, base of hair	vessel, ray cells next to vessel, periderm	yes
		pith ray cell surrounding inner of xylem	phloem fibres	
pBIN2DB3 (-195)	12(12)	undetected	undetected	no
pBIN2DD1 (-110)	10(10)	undetected	undetected	no

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Claims

1. An isolated DNA sequence according to figure 1 B (SEQ.ID.NO.1) representing a *CCoAOMT* promoter or a functional part thereof having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel.

2. An isolated DNA sequence according to figure 1 C (SEQ.ID.NO.2) representing a *CCoAOMT* promoter or a functional part thereof having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel.

3. An isolated DNA sequence according to claim 1 or 2 representing a *CCoAOMT* promoter or a functional part thereof having said biological activity wherein the cells adjacent to the vessel are xylem ray cells.

4. An isolated DNA sequence according to claim 1, 2 or 3 representing a *CCoAOMT* promoter or a functional part thereof having said biological activity wherein the vessel is a differentiating vessel.

5. A promoter sequence conferring expression in living vessel elements and/or in ray cells, adjacent to living or autolysed vessel elements and/or in fibers adjacent to said living or autolysed vessel elements, with the proviso that said expression does not occur constitutively in xylem fiber cells or ray cells that are not adjacent to said vessel elements.

6. An isolated DNA sequence according to claim 1 wherein the functional part represents the region from -114 to - 456, preferably from -199 to - 456 according to the numbering indicated in figure 1B.

7. An isolated DNA sequence according to claim 2 wherein the functional part represents the region from - 110 to - 497, preferably from - 195 to - 497 according to the numbering indicated in figure 1C.

8. A recombinant DNA comprising any of the isolated DNA sequences according to claim 1 and/or 2.

9. A plant or tree cell comprising a recombinant DNA according to claim 8 integrated

in its genome.

10. A transgenic plant or tree comprising the cell according to claim 9 and its progeny thereof.

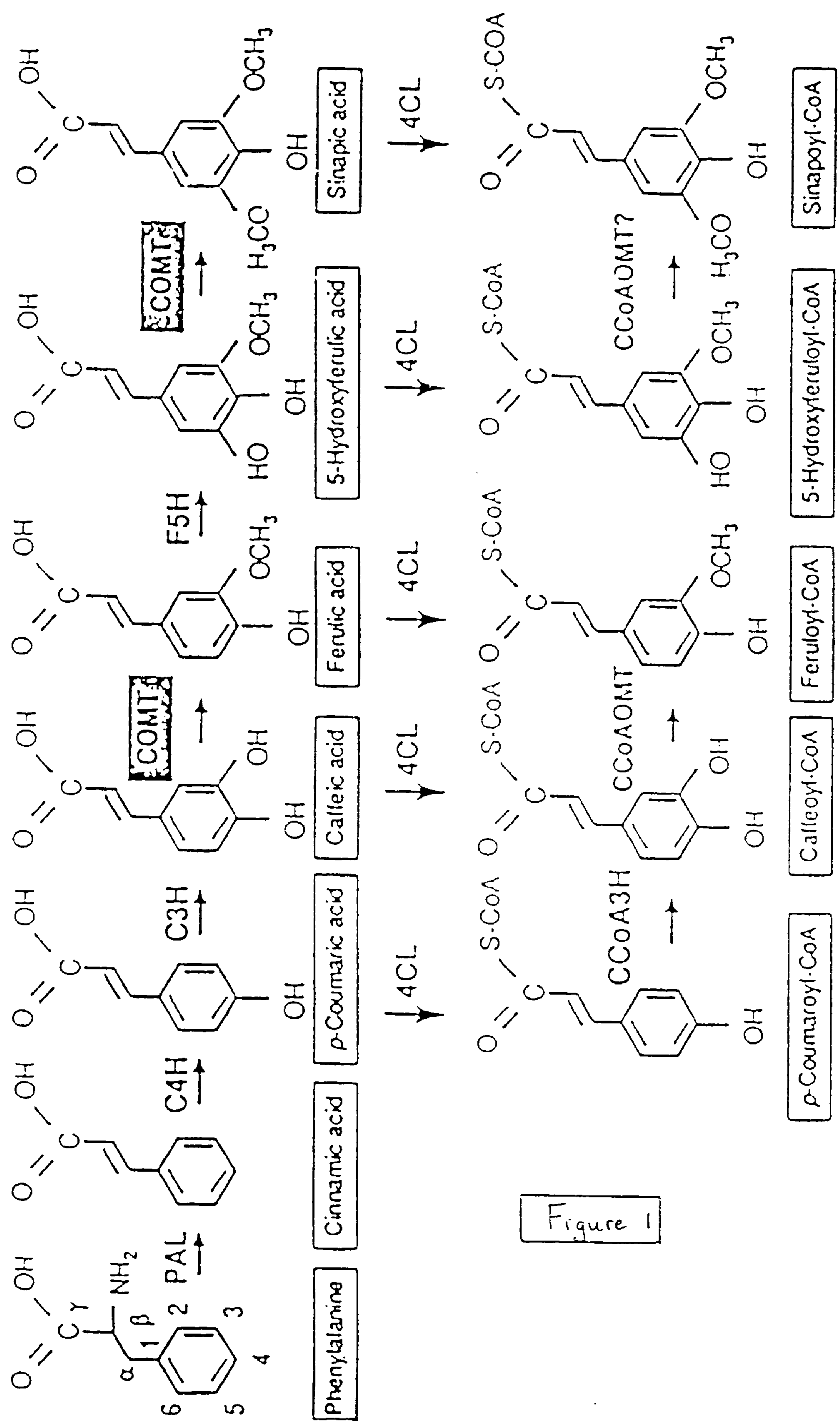
Phenylalanine
PAL
Cinnamic acid
C4H
p-Coumaric acid
C3H
Caffeic acid
COMT
Ferulic acid
F5H
5-Hydroxyferulic acid
COMT
Sinapic acid
4CL
p-Coumaroyl-CoA
CCoA3H
Caffeoyl-CoA
CCoAOMT
Feruloyl-CoA
5-Hydroxyferuloyl-CoA
CCoAOMT?
Sinapoyl-CoA

Figure 1

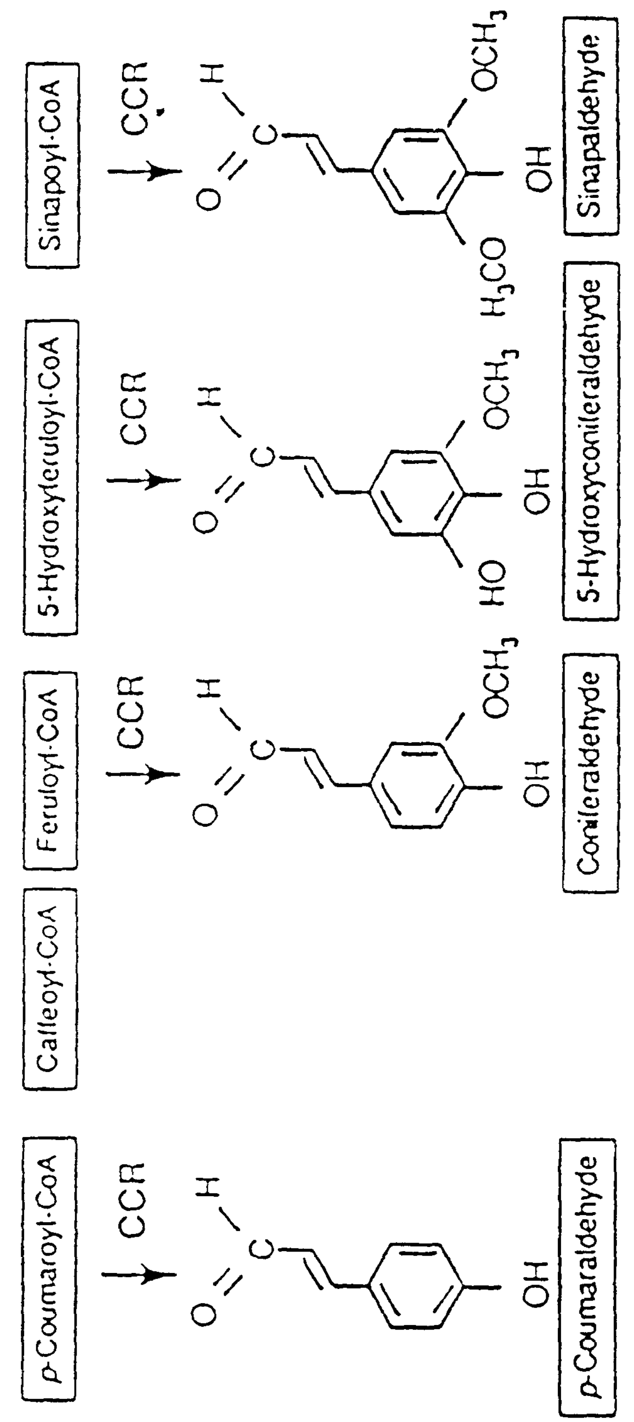

Figure 1 continued

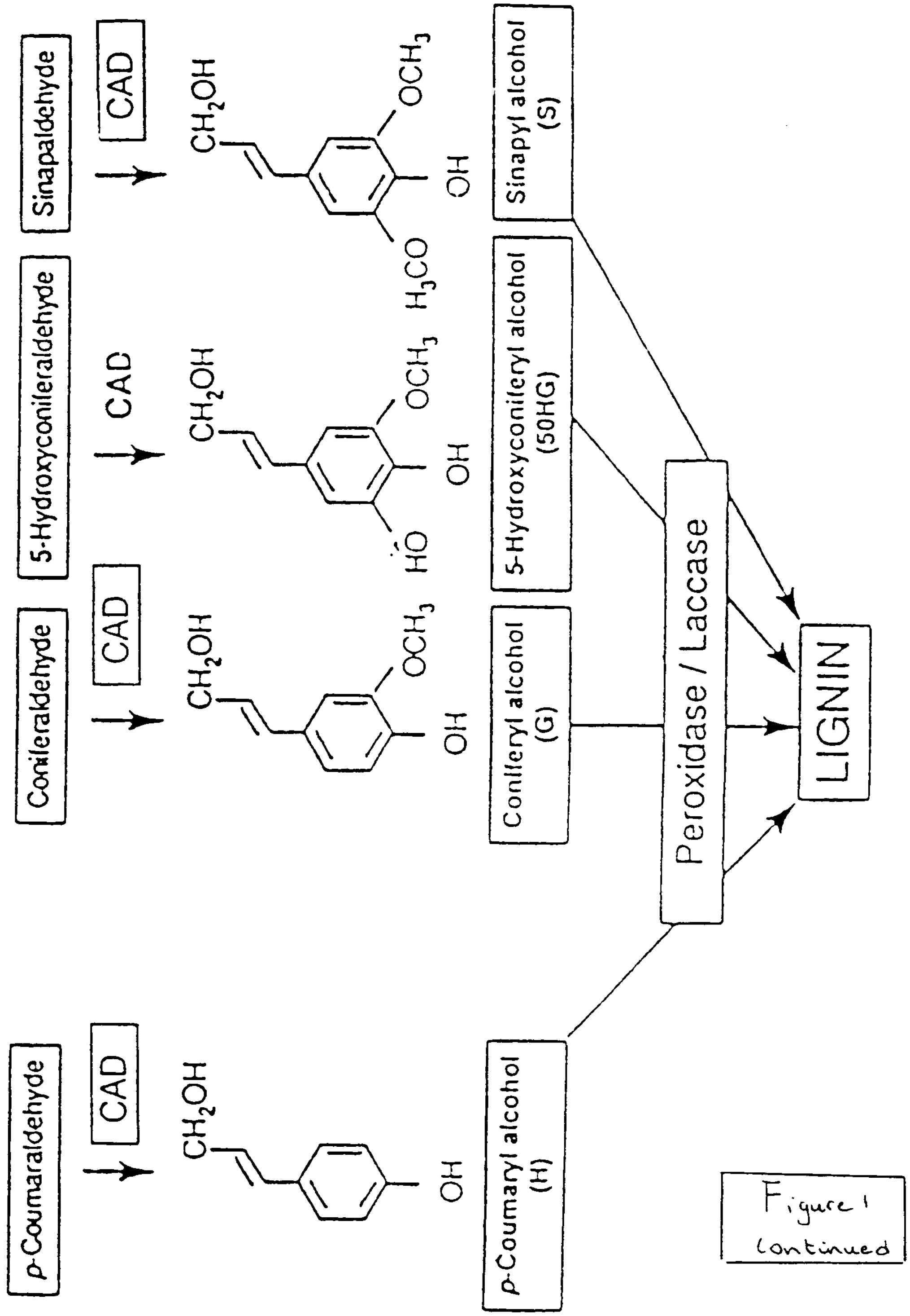

Figure 1 continued

Figure 1. The lignin biosynthesis pathway.

PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; C3H, coumarate-3-hydroxylase; COMT, bispecific caffeic acid/5-OH-ferulic acid-*O*-methyltransferase; F5H, ferulate-5-hydroxylase; CCoA3H, coumaroyl-CoA-3-hydroxylase; CCoAOMT, caffeoyl-CoA-*O*-methyltransferase; 4CL, 4-OH-cinnamate-CoA ligase; CCR, cinnamoyl-CoA-reductase; CAD, cinnamyl alcohol dehydrogenase.

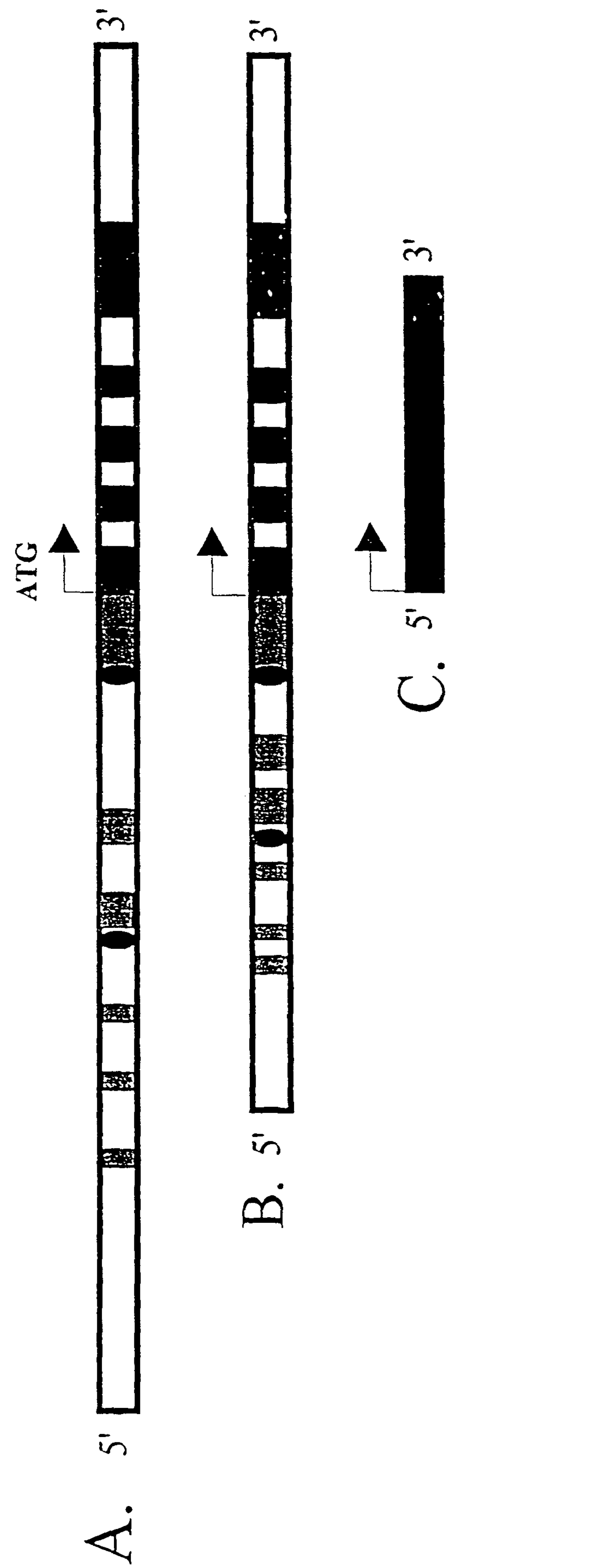

Figure 1-A. Structure of CCoAOMT genes.
A: CCoAOMT gene1, *pop1*. B: CCoAOMT gene2, *pop2*.
C: cDNA.

● AC-element ▩ Homologous region ■ Exon

Figure 1-B. DNA sequence of *PtCCoAOMT1* (AJ223621), from *P.trichocarpa* cv. trichobel

The coding regions are indicated as capital letters. The putative boxes such as AC-II and TATA are underlined. The primers used to generate the deletions of the promoter are indicated in *italics*.

-1990 -1970 -1950 -1930 -1910 -1890

tctagagttcgtggtttattttatattttaaagtatttaaaaaaaaataatttttttaatttttatttggtttaaattaatttttttttgtatttttatattattttgatgtgatga

-1870 -1850 -1830 -1810 -1790 -1770

tataaaaaataaaattttaaaagtaacccttgtctaaatacaaaataggctctatatataagccagaggaggtgatattttgattattttctttaagactacagatgacccgataacatg

-1750 -1730 -1710 -1690 -1670 -1650

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-1630 -1610 -1590 -1570 -1550 -1530

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-430 -410 -390 -370 -350 -330

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                                                                                                        AC-II element
-190        -170        -150        -130        -110        -90
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                                                                   TATA
-70         -50         -30         -10         10          30
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↓ Transcription start site                                                ↓ Translation start site
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290         310         330         350         370         390
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770         790         810         830         850         870
CAAATGATTGAAGATgtaagaaatactctatgttcgacaaaaaatgaaaatggaagagggaaaaacatcctttttttgtctacttgtatgagaaaaagaaacgatgctcgtttttttaactt
890         910         930         950         970         990
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1010        1030        1050        1070        1090        1110
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                                ↓Poly A cleavage site
1490                1510                1530                1550                1570                1590
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1730                1750                1770                1790
agtttagagacagacattgaggttgtggcgaggaaaatccgaaaattatcttgtaaacaagtctaacttttcacacgaat
```

Figure 1-C. DNA sequence of *PtCCoAOMT2* (AJ223620), from *P. trichocarpa* cv trichobel. The coding regions are indicated as capital letters. The TATA-box and AC-II elements are underlined. The primers used to generate the deletions of the promoter are indicated in *italics*.

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-760 -740 -720 -700 -680 -660

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-520 -500 -480 -460 -440 -420

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AC-II element

-400 -380 -360 -340 -320 -300

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-280 -260 -240 -220 -200 -180

agaaattgaattagtgtccgaaacctaaaatgaccactggacaaacaccgataagtgggtcccaaaatacccacggtgtcctaaga***actcaccaacccc***caccccggctggaagccggtcc

AC-II element

-160 -140 -120 -100 -80 -60

aaccaccccactactccggctcaaaccggactctcatctccaataaataccacc*tgcccttgccatttt*caatcaggtcagacatccttaccatcgtcgcccccagaaaaaccttccaac

TATA ↓ Transcription start site

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↓ Translation start site

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200 220 240 260 280 300

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320 340 360 380 400 420

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680 700 720 740 760 780

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1160 1180 1200 1220 1240 1260

GGATTGAAATCTGCATGCTTCCTGTTGGTGATGGTATCACTCTCTGCCGTCGGATCAAGtgaggggctgcattccctgccgatattatatatatatcaaatgttgaccactgatttggtc

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                       Poly A signal

1400        1420        1440        1460        1480        1500
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                                 ↓ Poly A cleavage site

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1640        1660        1680        1700
gctaaccgcaagtaaaagaatgtgtatcgagaaagggcttttgcaagcttggccttgaactagcgtggaaaa
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1151	
prom1-2.msf{pccoaomt2}	TT TTTATATA TTTGTCCTGT GATTATCAT. .... AGACGGTAA
prom1-2.msf{pccoaomt1}	TATATGCATA CCAAGTATAC CCTTTACATC TGCCCTAATT AAGACTGTAA
Consensus	T -T -T-- ATA ------- T-- -- TT--CAT- ---------- -AGAC- GTAA
	Upper primer
1201	
prom1-2.msf{pccoaomt2}	AACGAAATTG GATTTTT.. .... TT ATTTTGTTGG AGAAAAAAAA
prom1-2.msf{pccoaomt1}	AACGGATTTG GATTTTTGCC GACAAAGGCT AGTTTG .TGG AGAAAAAACA
Consensus	AACG -A- TTG GATTTTT--- -------- -T A- TTTG- TGG AGAAAAAA - A
1251	
prom1-2.msf{pccoaomt2}	AAGAAAATAA ATATTGTCAG CAGTAAGAC. ....G GAGAGATTCT
prom1-2.msf{pccoaomt1}	ACGAAGATAA ATTTTTAGAT GACAAAGTCA ACAATAGTTC GAGAGATTCT
Consensus	A -GAA -ATAA AT -TT--- A- -A- -AAG- C- ------- - GAGAGATTCT
1301	
prom1-2.msf{pccoaomt2}	TAAAAGGAGT CATCCATTGT CAATGCGGTG GCTACGAGCC ACCAACTCCC
prom1-2.msf{pccoaomt1}	TTAAAGGACT CATCCGTTG. .ACGGAGGTG GCCATATGCT ACCAACTCTT
Consensus	T- AAAGGA- T CATCC- TTG- - A- -G - GGTG GC- A--- GC- ACCAACTC--
1351	
prom1-2.msf{pccoaomt2}	GTGGAGTCAA ATTCTTGAGG ACACCTCACC AACCCCTTAC CCACTTTCTA
prom1-2.msf{pccoaomt1}	G .GACGTGGA GTCCCTTTGG TAATTTCACC TATCCCTCAC CCAATTTCTA
Consensus	G- G-- GT- A - T- C- T-- GG - -A-- TCACC -A -CCCT- AC CCA- TTTCTA
1401	
prom1-2.msf{pccoaomt2}	TTAGC.. A GCACATGTA .... GCCATCCCCA ACAACAAAGT
prom1-2.msf{pccoaomt1}	TTAGCAGTTA GCACATGTAA TTTATGATTG GTGAGCCCAG CACAAATCTT
Consensus	TTAGC--- -A GCACATGTA- ---------- G-- A- CCC-- --- A- A--- T

Fig 1_d

```
1451
prom1-2.msf{pccoaomt2}  GGTGAGCCCA CCACA..... .......              ... ... ...ATTT TCTACTCTCT
prom1-2.msf{pccoaomt1}  TT CCAGTTAA ACACATATAT TAATTTATGA TTAATTATTT AATTCTCTCC
Consensus        -       ---AG-   --A -  CACA----- ---------- ------          ATTT -   -T- CTCTC-

1501
prom1-2.msf{pccoaomt2}  ACGATT.   .TA AATCAATTAC ACGTGGCATA AAATGTCGAG CCTTTTATTT
prom1-2.msf{pccoaomt1}  ACTCTTAACA AATTAATCAT ACATGGCATA ACAT  .TTTAG CTTTTGATCT
Consensus               AC   --TT---   A AAT -AAT- A-   AC -TGGCATA A-  AT-  T-   -AG C- TTT -AT- T

1551
prom1-2.msf{pccoaomt2}  CAAGAAACCA AACCTAACAC CGTGAACTTA ATTTCT. TTC GCAAATATCT
prom1-2.msf{pccoaomt1}  CGAGAATCTC  TACCTA..   AC CATTGACTTC TTTACTGTTC AGGAATCTTA
Consensus               C -AGAA- C--    -ACCTA-    -AC C- T--   ACTT-    -TT  CT-  TTC ---    AAT  -T --
1601
prom1-2.msf{pccoaomt2}  AGAAATTGAA TTAGTGTC..   CGAAACCTAA AATGAC.... ..........
prom1-2.msf{pccoaomt1}  GAACCGATAT TTGGTGTCAT AGATCCCAAA AATGACGCCA GCGATGCCTA
Consensus                  -A----        -A-  TT- GTGTC--      -GA  --CC  -AA AATGAC---- ----------

1701
prom1-2.msf{pccoaomt2}  .......... .......... ....                    .CACT GGACAAACAC CGATAA  GTG
prom1-2.msf{pccoaomt1}  AGGGAAGGAG TACCACTAGC CCACAGCACG ATACG ATCAC CAACAAGGTC
Consensus                             ---------- ---------- ------CAC- -    -AC   -A -CAC C-  A -AA  -GTG

1751
prom1-2.msf{pccoaomt2}  GGTCCCA.. .........           . AAATACCCAC GGTGTCCTAA C.........
prom1-2.msf{pccoaomt1}  GGTCCCATAT TTGGTGGGCC AAAAACCCAC ATTATCCTTC GTCCTAACTA
Consensus               GGTCCCA--- ----------          AAA- ACCCAC -  T-    TCCT--   G---------
```

1801
prom1-2.msf{pccoaomt2} .... AACTC ACCAACCCCC ACCCGGTTGG AAGCCGGTCC AACCACCCCA
prom1-2.msf{pccoaomt1} CAGGA ACCTC ACCAACCCCC TCCCGGTTGG TAGCCGGTCC AGCCTCCCCG
Consensus -----A -CTC ACCAACCCCC -CCCGGTTGG -AGCCGGTCC A CC- CCCC-

1851
prom1-2.msf{pccoaomt2} CTACTCCGGC TCAAACCGGA CTCTCATCTC CAATAAATAC CACCTGCCC.
prom1-2.msf{pccoaomt1} CTACTCCAAT TCAAACCGGG CTCTCATTTC CAATAAATAC CACCCGCCCT
Consensus CTACTCC--- TCAAACCGG- CTCTCAT- TC CAATAAATAC CACC -GCCC-

1901
prom1-2.msf{pccoaomt2} TTGCCATTTT CAATCAGGTC AGACATCCTT ACCATCGTCG CC.. .CCCAG
prom1-2.msf{pccoaomt1} TTACCATTTT CGATCAGGTT AGGCATCACT ACCATCATCA ACAAAAAAAA
Consensus TT -CCATTTT C- ATCAGGT- AG -CATC- -T ACCATC- TC- -C------ A-

1951 2000
prom1-2.msf{pccoaomt2} AAAAACCT TC CAACGCCAGG AAAGAGAGTA TAGTTTTGTT ATAAGATATA
prom1-2.msf{pccoaomt1} AAAAAAAATC CAAGGCCAAG AAAGAGATCG TAGTTTAATT AGAAGATATA
Consensus AAAAA-- -TC CAA- GCCA -G AAAGAGA-- - TAGTTT-- TT A -AAGATATA

2001
prom1-2.msf{pccoaomt2} CAAAATA**ATG GCCGCCAACG GAGAGGAACA GCAGACTCAG**
prom1-2.msf{pccoaomt1} CACAATA**ATG CCCACCAACG GAGAGGAACA GCAAAGCCAG**
Consensus CA -AATA**ATG .. CC.. CCAACG GAGAGGAACA GCA.. A .. ..CAG**

Lower primer

Figure1-D. Comparison of DNA sequences of the two promoters of CCoAOMT genes from popar. The upper line, pccoaomt2, is promoter of pop2. The lower line, pccoaomt1, is promoter of pop1. The part of coding sequence is in bold type. Conserved promoter regions are indicated by open boxes. The AC-element II are marked underline. The sequence represented by horizontal arrows are chosen as primers for amplifying promoters from different species in popar. The vertical arrow is indicated translation start site.

⟨End of Fig. 1-D⟩

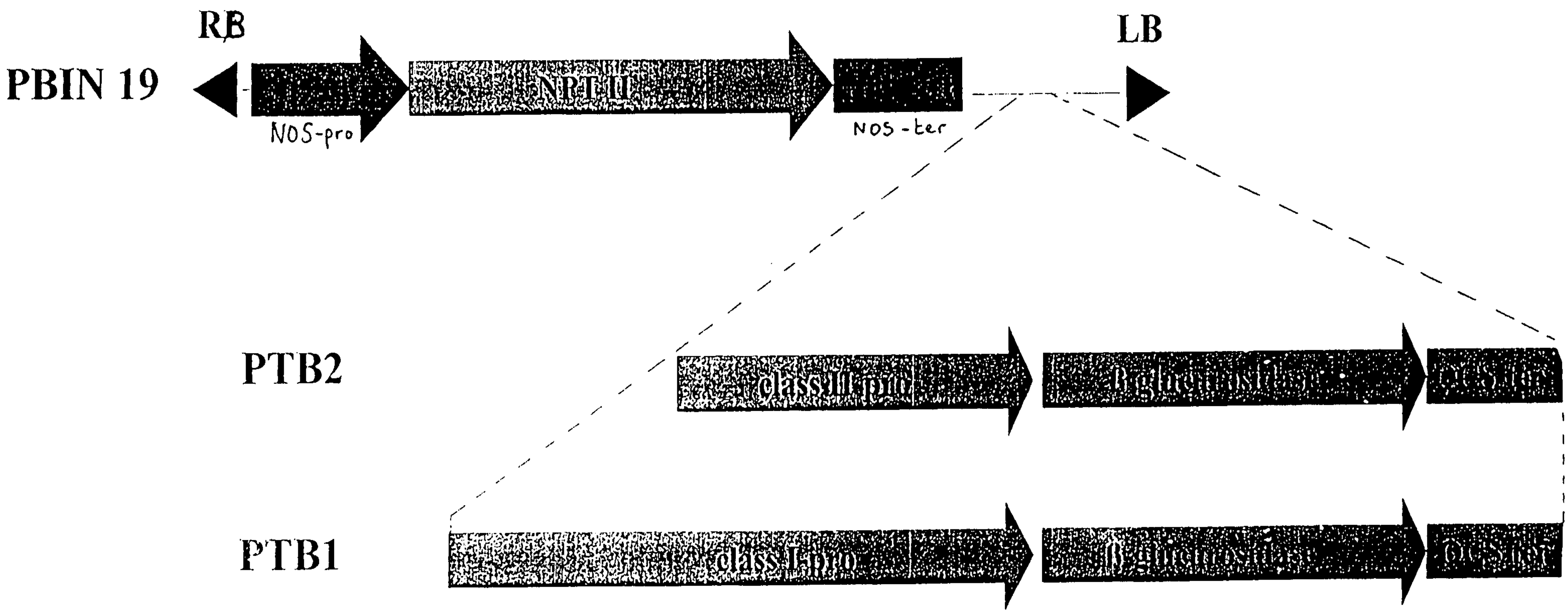


Fig. 2. Structure of expression vectors

PTB1: chimaeric CCoAOMT class I promoter-gus cassette.
PTB2: chimaeric CCoAOMT class II promoter-gus cassette.

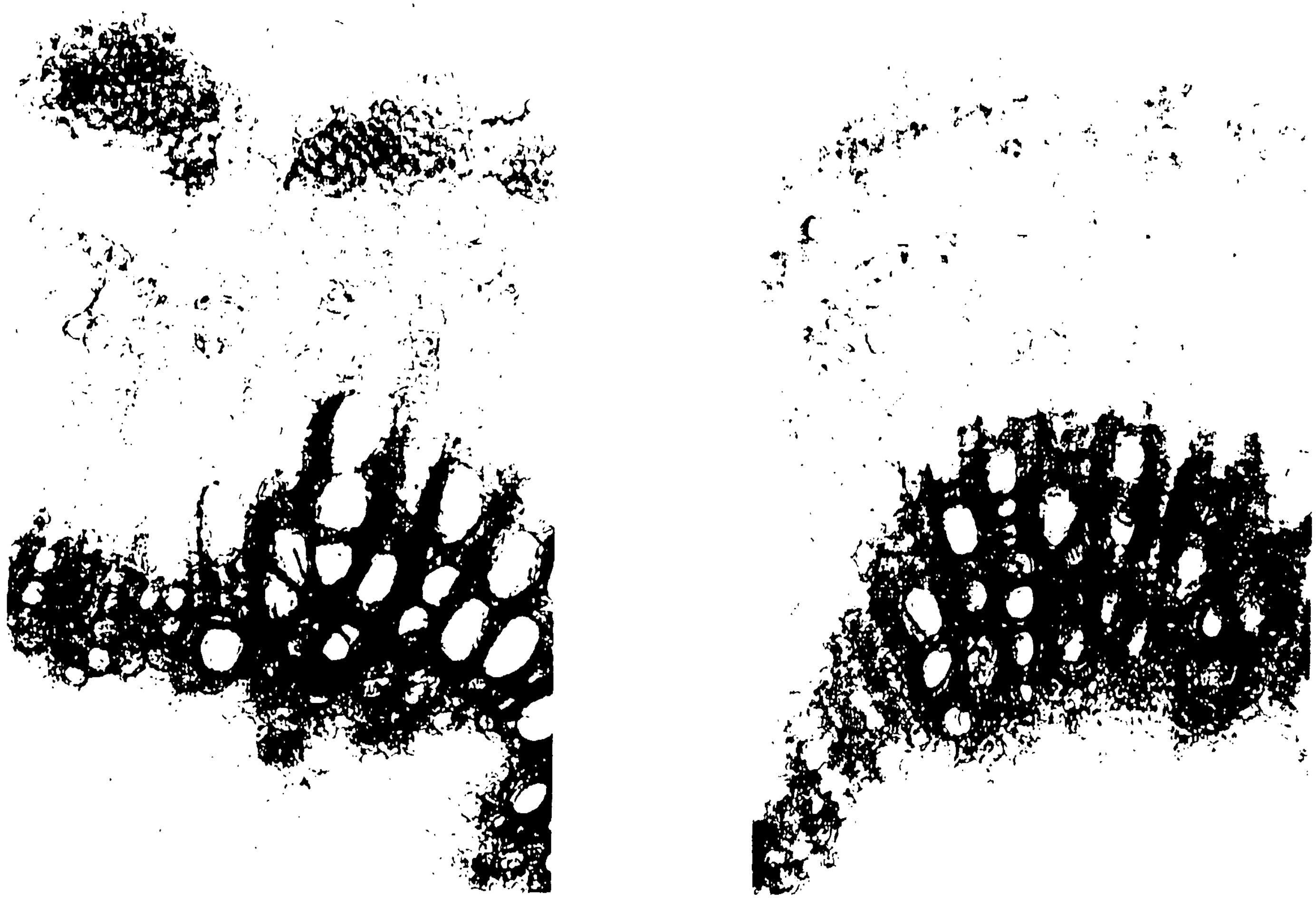


Fig. 3.

Histochemical localization of GUS activity in transgenic plant

The cross section of young internode were double-stained . Lignified cells and tissues are revealed by red color after phloroglucinol-HCl staining. GUS activity is only detected in the xylem ray cells located between the lignified vessels.

Fig • 4. Histochemical localization of GUS activity in transgenic poplar. The transverse section through young stem shown GUS activity localized in vessels and in the ray cells surrounding and between vessels.

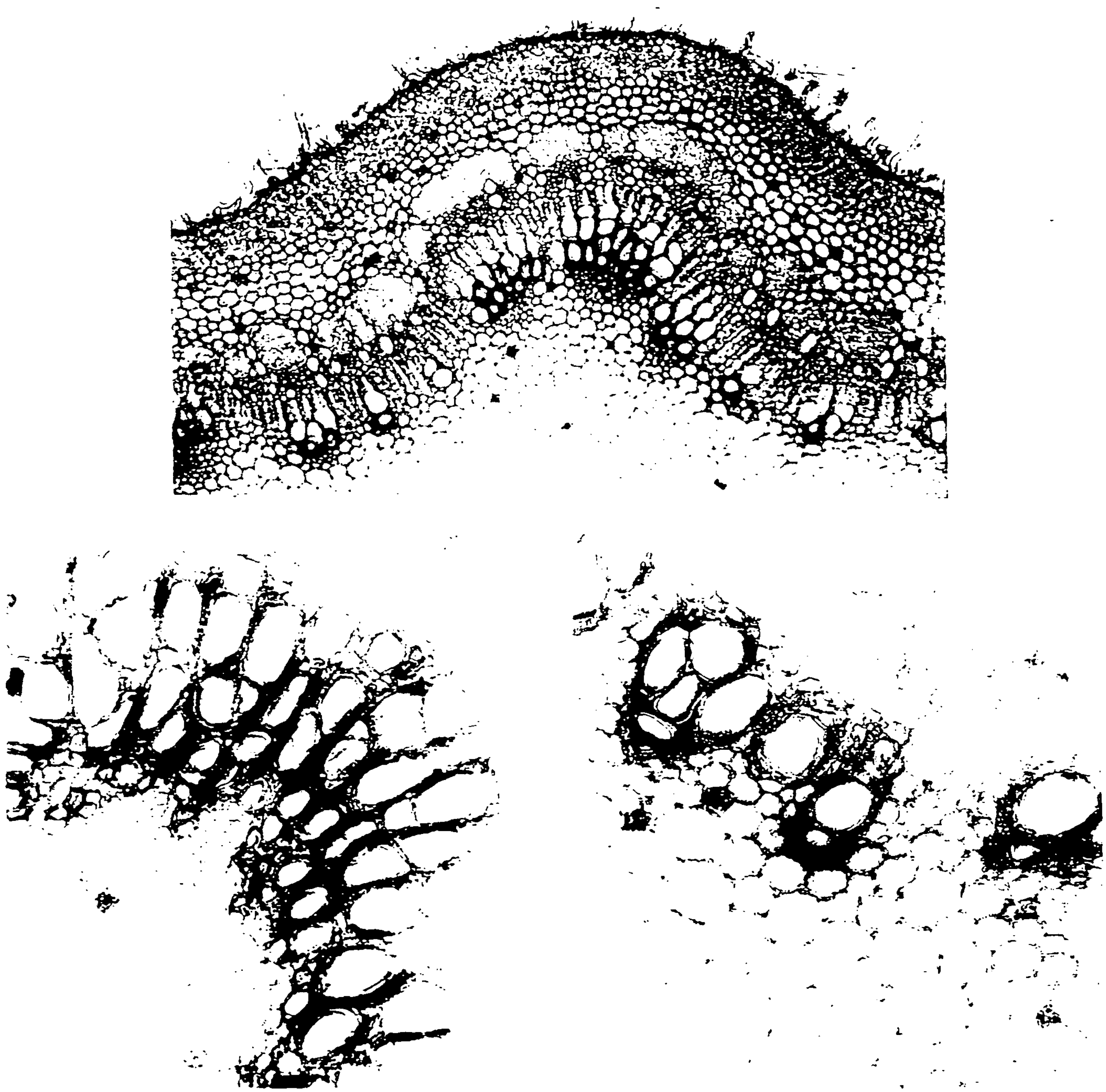
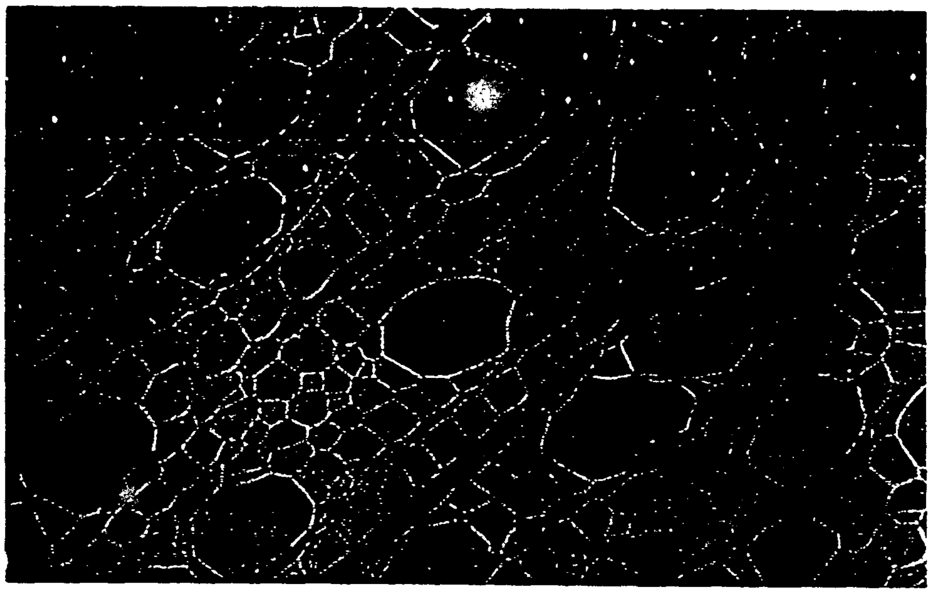


Fig. 5. Histochemical localization of GUS activity in transgenic poplar stems. The transverse sections shown GUS activity was localized in vessels and in the ray cells adjacent to vessels in xylem tissue of old stems: A, in pBINPOP1 transform; B, in pBINPOP2 transform.

A.



B.

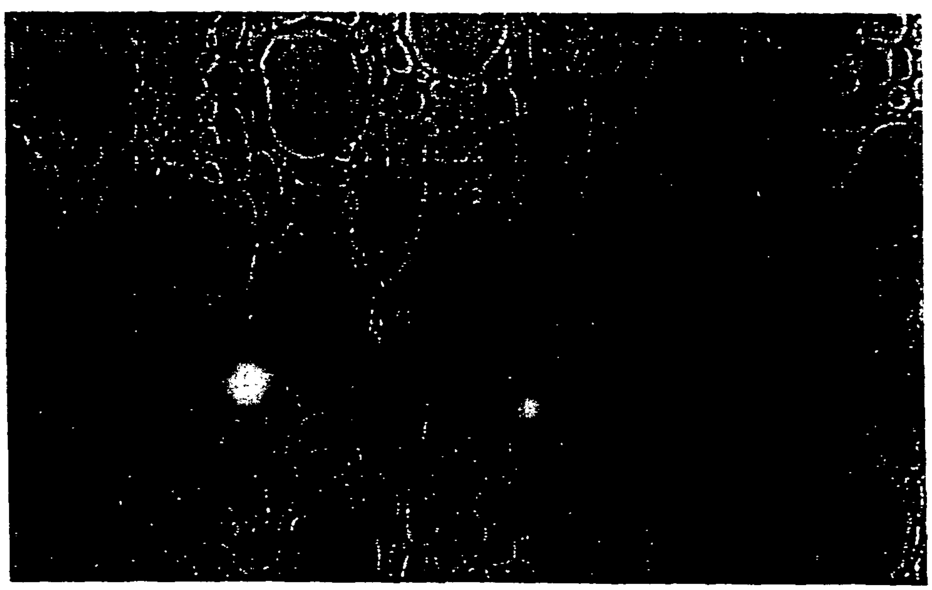
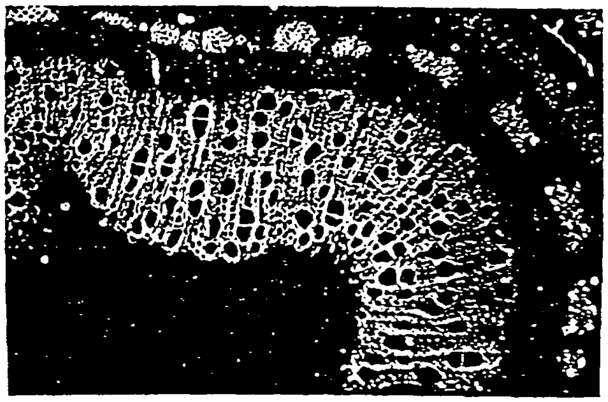


Fig • 6. Dark-field image of transverse sections through stem in transgenic poplar.
The red-purple represents GUS activity.

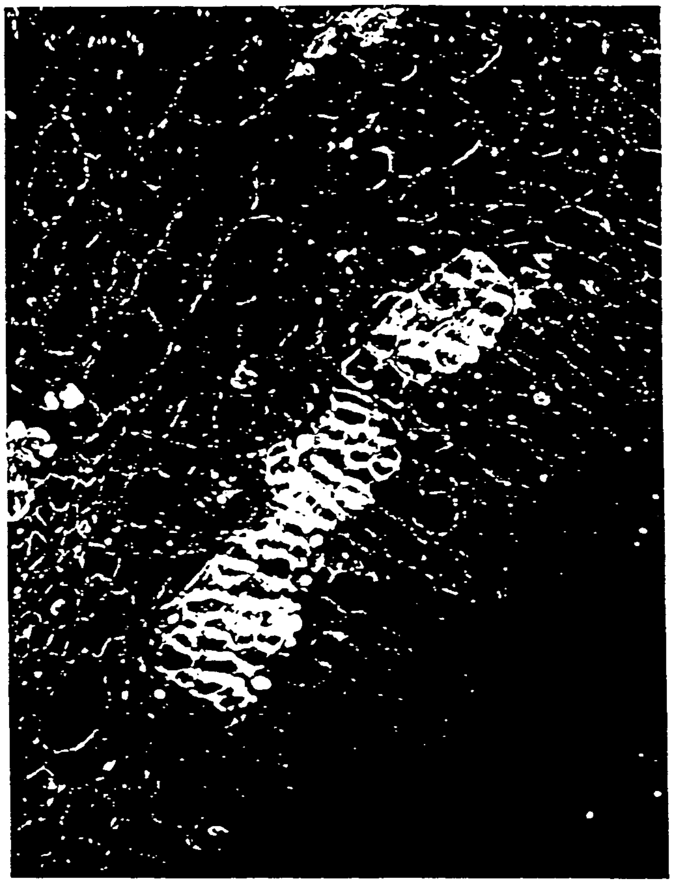


A. B. C.

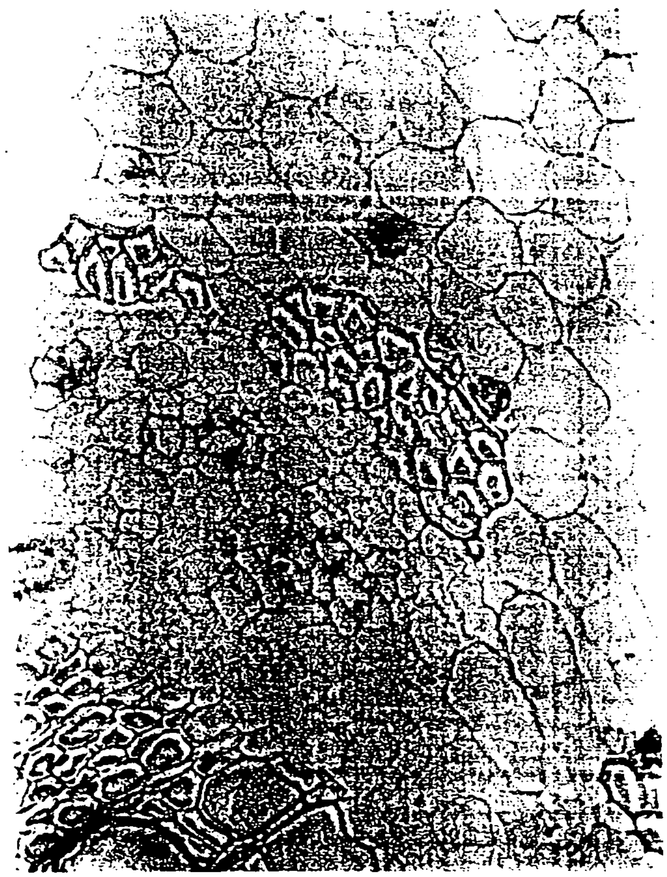
Fig. 7. Histochemical localization of GUS activity in transgenic poplar stems.
A, the section was stained with phloroglucinal and GUS, lignified walls stain red.
B, GUS activity localization with CCoAOMT2-GUS construct.
C, GUS activity localization with CCoAOMT1-GUS construct.

Fig. 8. Enlargement of transverse sections of stem shown GUS activity: A, in the companion cells; B, in the phloem fibres: and C, in periderm.

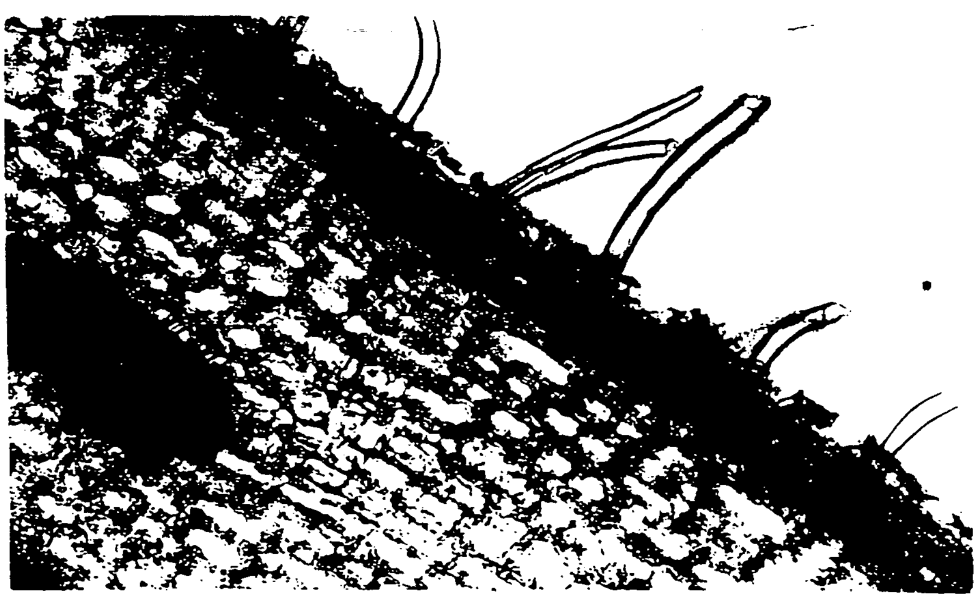
A.



B.



C.



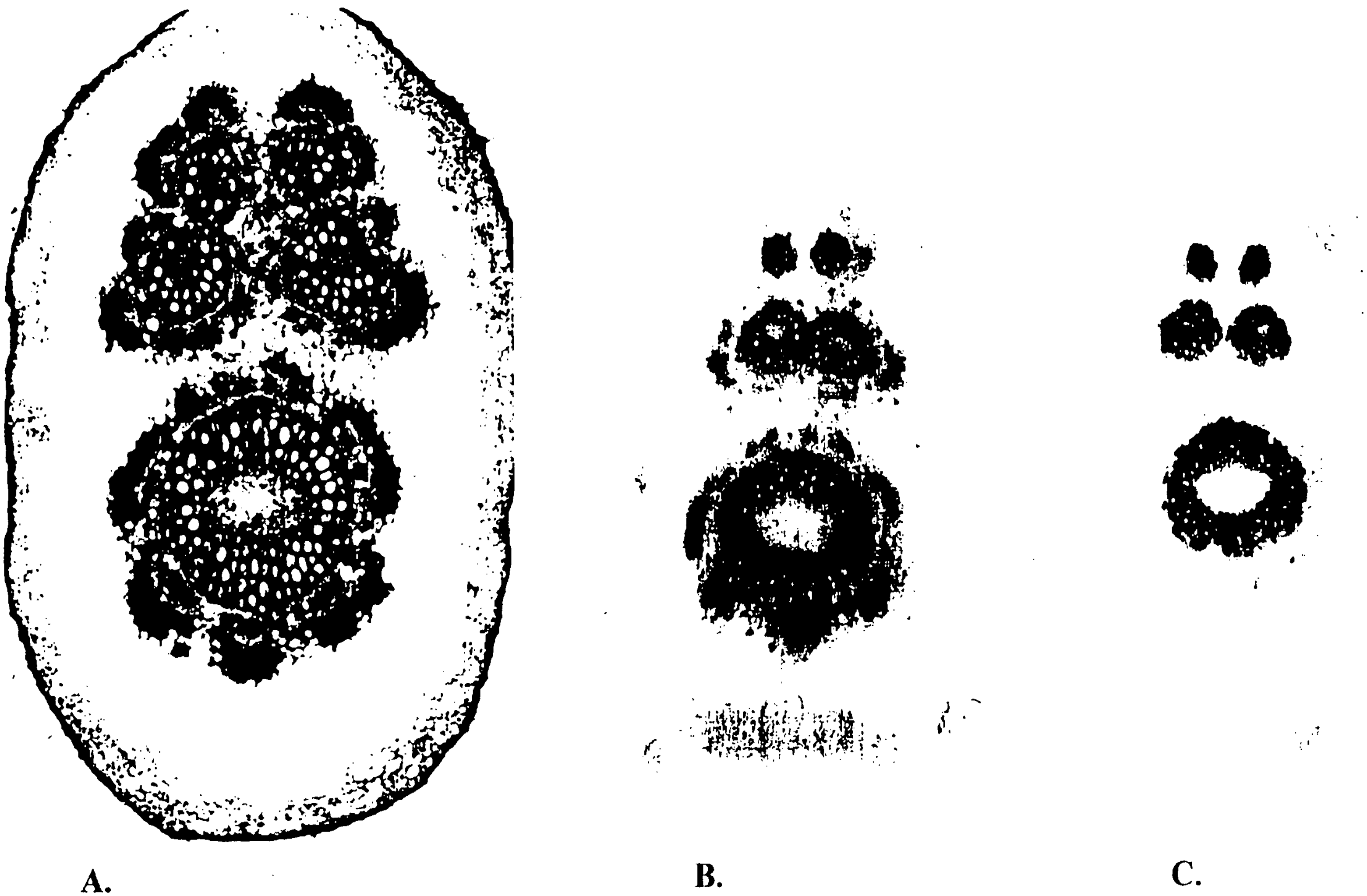
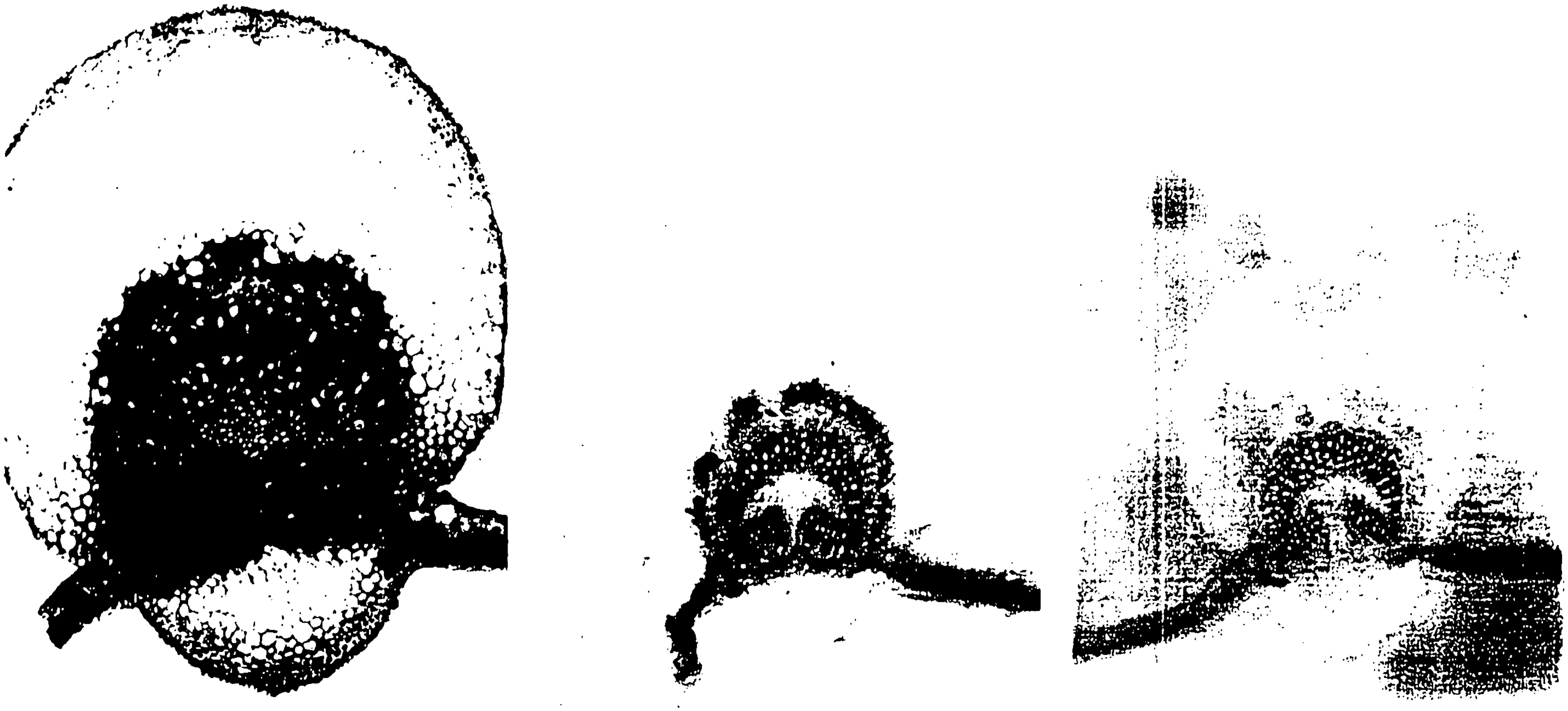


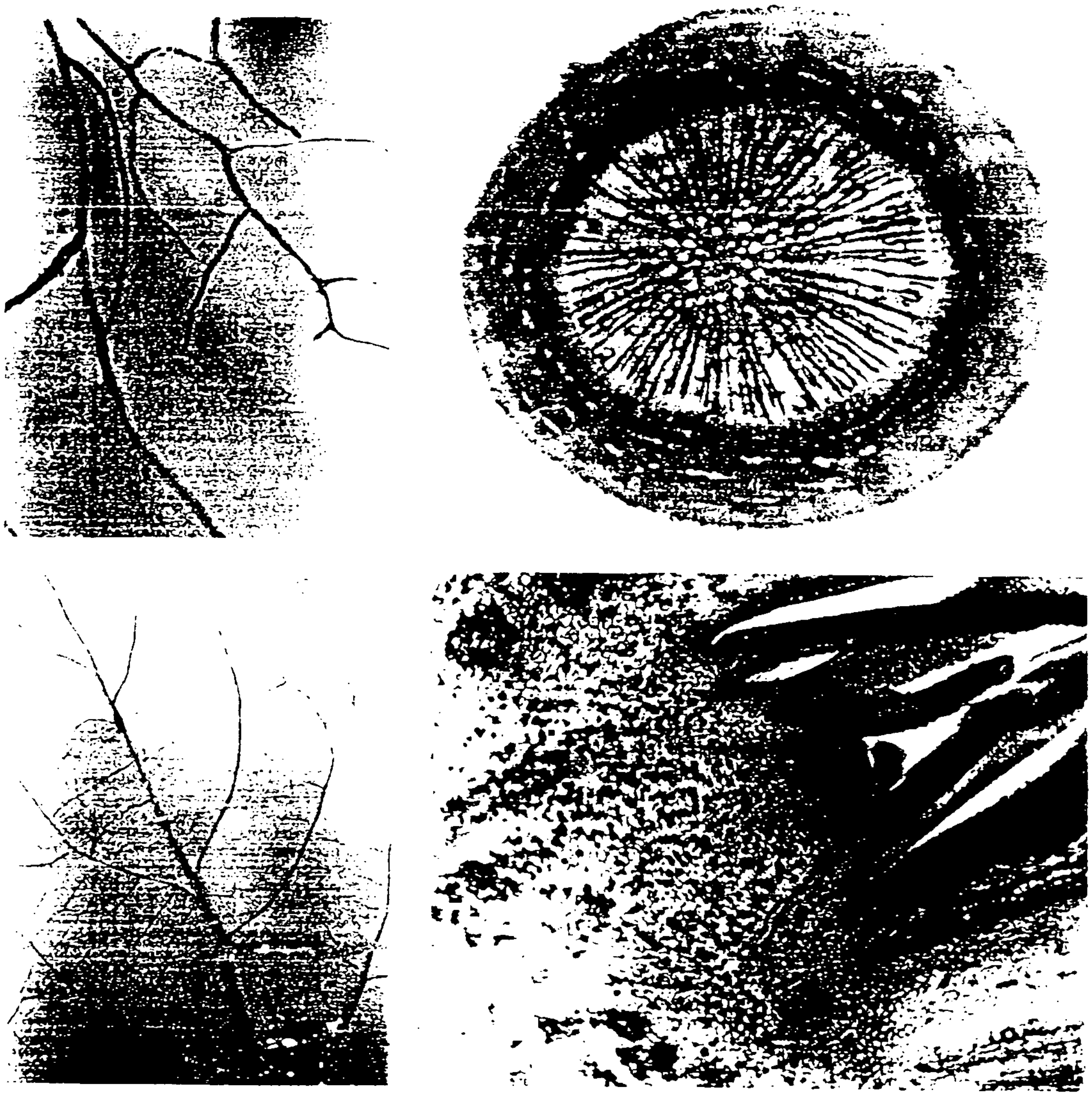
Fig. 9:
Histochemical localization of GUS activity in transgenic poplar petioles.
A: The section was stained with phloroglucinol; lignified walls stain red.
B: GUS activity localization with *CCoAOMT*2-GUS construct.
C: GUS activity localization with *CCoAOMT*1-GUS construct.



A. B. C.

Fig. 10
Histochemical localization of GUS activity in transgenic poplar leaves.
A: The section was stained with phloroglucinol; lignified walls stain red.
B: GUS activity localization with *CCoAOMT2*-GUS construct.
C: GUS activity localization with *CCoAOM*T1-GUS construct.

Figure 11. GUS staining in transgenic poplar.
A and B, in root; C, in meristem of shoot bud; D, in leaf.



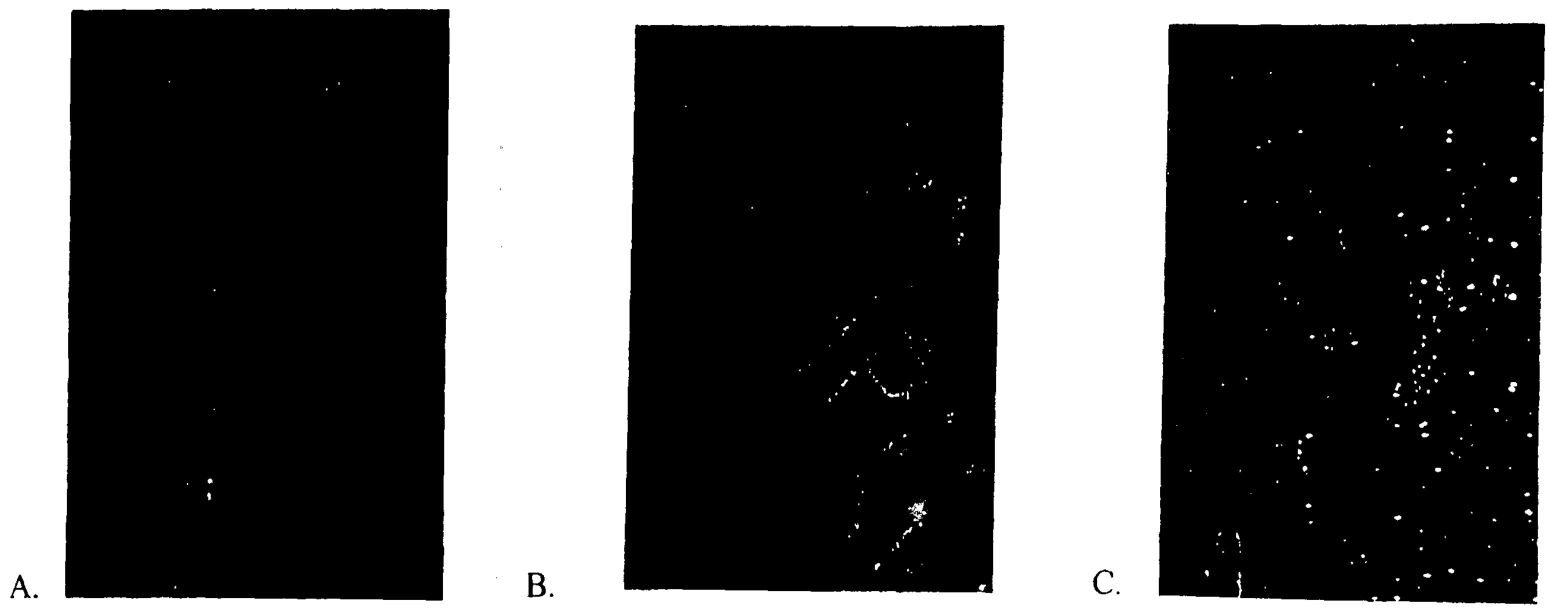


Fig. 12. Immunolocalization of CCoAOMT in the stems of *P. trimula x P. alba*.
CCoAOMT was localized intensively in the xylem ray cells adjacent to vessels and in vessels (B), and in the phloem fibres (C). A, control reaction.

Fig. 13. DNA sequences of CCoAOMT genes from different species in poplar.

pgdel: *P. deltoides* S 9-2 B IBW
pgalb: *P. alba*
pgcil: *P. ciliata* 72085 I
pgnig: *P. nigra italica aral*
pgmig: *P. nigra Ghoy* B IBW
pbdel: *P. deltoides* S 336-4 B IBW
pgeup: *P. euphratica;*
pglau: *P. laurifolia* 69/65
pgen: *P. trichocarpa* vc trichobel (this invention)
number 1 and 2: refer to *PtCCoAOMT1* and *PtCCoAOMT2*, respectively
a, b and c: different alleles.
1D1: *pBIN1DD1*; 1D2: *pBIN1DC2*; 1D3: *pBIN1DB3*; 1D5: *pBIN1DA5*; 2D1: *pBIN2DD1*; 2D3: *pBIN2DB3*; 2D4: *pBIN2DA4*

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                                   a                                                  c        cA gAA      A g    a


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Consensus                 -------TTC ACC-ATCCCT CACCCAATTT ---------- ---------- T----AT--- --------A- --GGTGAG-C CAGCACA--- ----------
                                 CC       C t T   T C C                                                   C
                                 ↑ 2D4 (AC-II)

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Consensus                 ---------- ---------- ---------- ----ATTCTC TCC-CTCT-- AC-AATT-AA T-A---A-A- --GGCAT--- AT-T---G-- --T-T-AT-T
                                                               T      A    c           t
                                                                                                        (XhoI) ↑ 1D5
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                                                              C ..  t g

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                                                  A    A           A a  C                                                             C

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               Consensus   ACTAG----- --------AC ----A-C--- AA-GTGGGTC CCA------- ------A-A- -C---C--T- --------CC T--------G -A-CTCACCA
                               G                                A        G                                                       c t
                                                           (BstXI) ↑                                                  (oligo 18) ↑ ID3/2D3

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                                   T          A            A A A  G A. G    GGC        tA A       ggC  TG           g g
                               ↑ D2 (oligo 19)                                                                                  ↑ 1D1/2D1(oligo 33)


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                                                     TG
                                                   +1 Transcription start site


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pileup_49.msf{pccoaomt2} aatggccgcc aacggagagg aacagca
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                          Translation start site
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Fig. 14. *CCoAOMT* promoter-GUS constructs

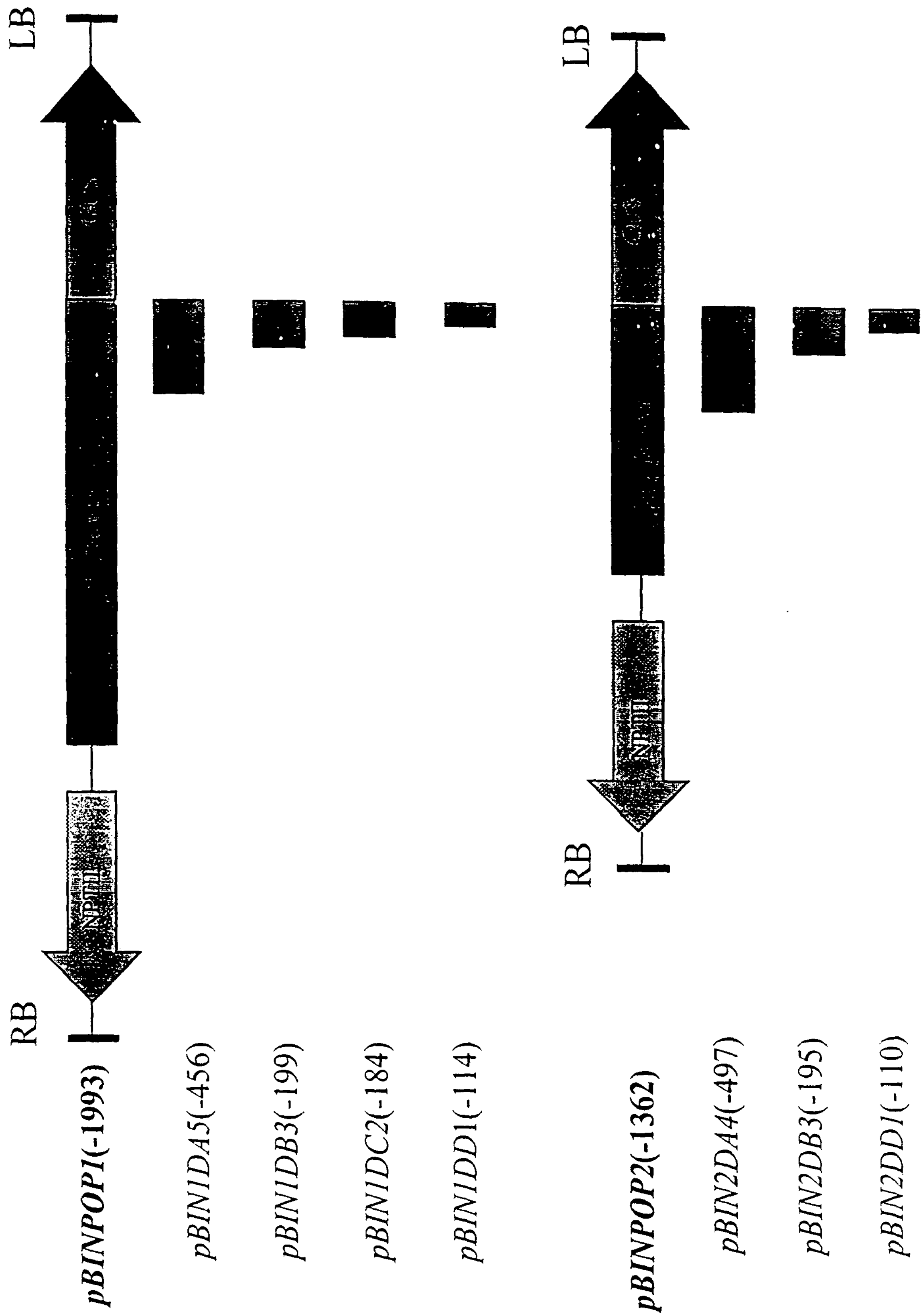


Fig. 15. Expression pattern of *pBIN1DC2* (-185) in transgenic poplar.

GUS activity was detected in periderm, cortex and phloem fibres.

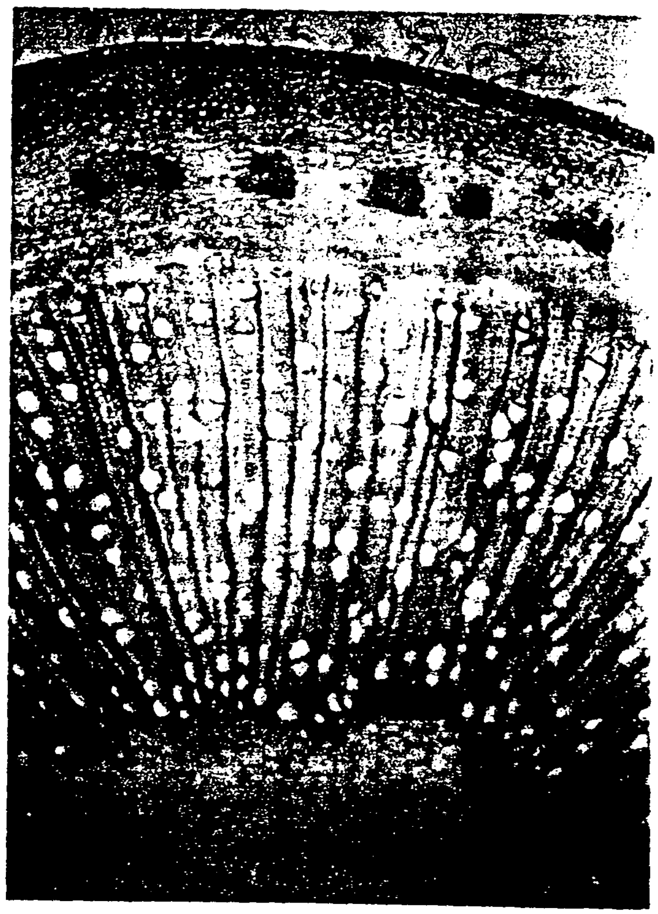
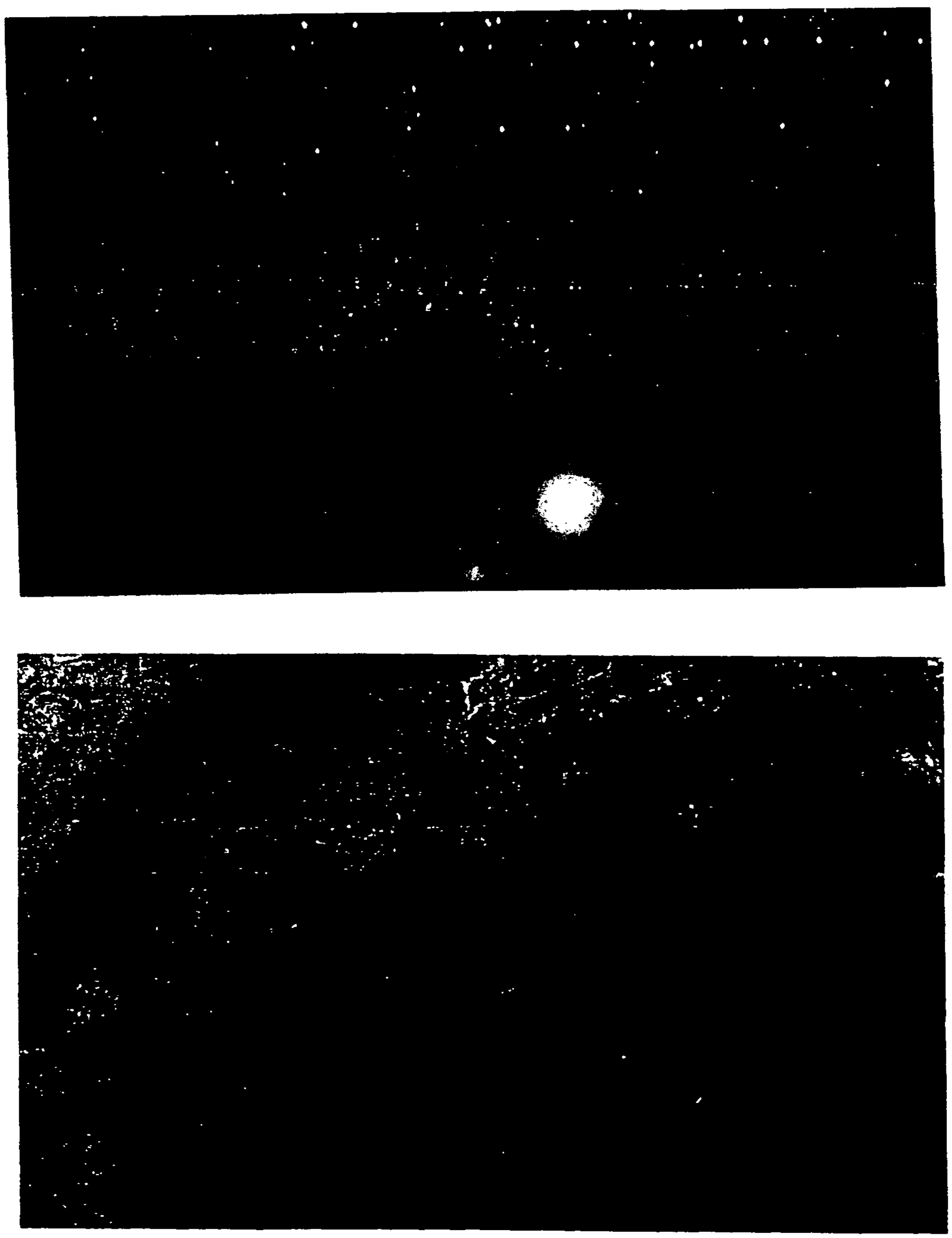


Fig. 16. Expression pattern of *pBIN1DA5* in transgenic poplar.

GUS activity was found in cambial ray cells, vessels and ray cells adjacent to the vessels.



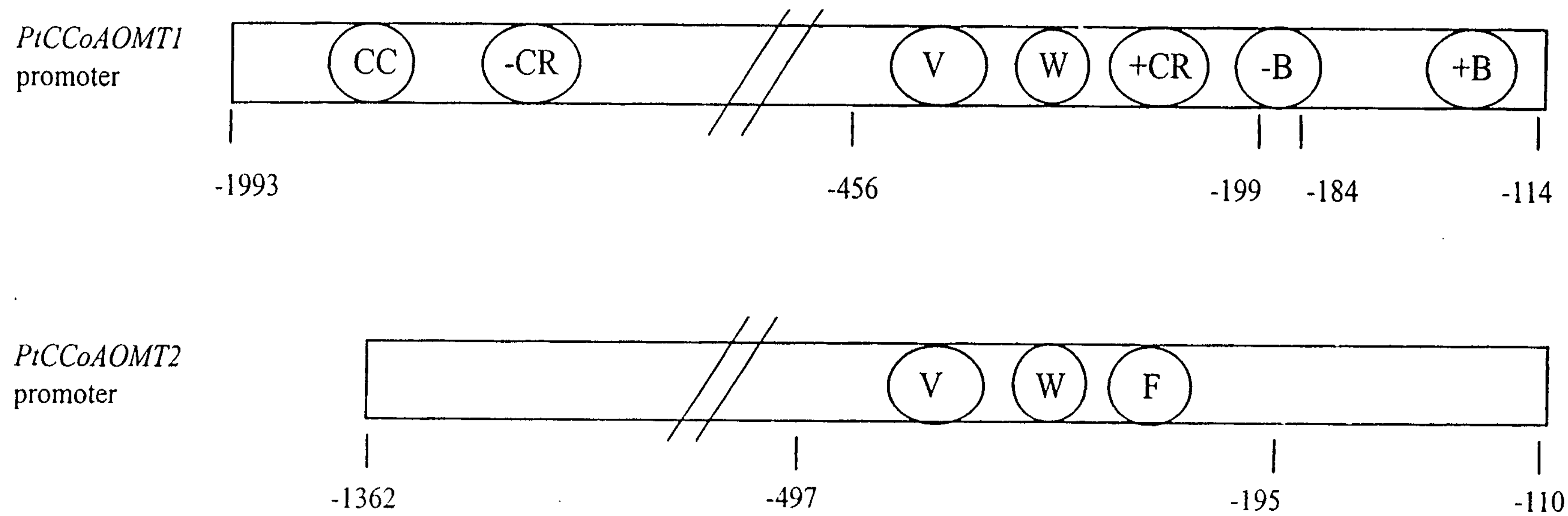


Fig. 17. A map of *CCoAOMT* promoters with domains as defined in this invention

+B: positive bark tissue domain; -B: AC-II element, negative bark tissue domain

+CR and -CR: positive and negative cambial ray cell domains

V: vessel and adjacent ray cell domain; CC: companion cell domain

F: phloem fibre domain

W: wounding domain

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Vlaamse Interuniversitair Instituut voor Biotechnologie
(B) STREET: Rijvisschestraat 118
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(ii) TITLE OF INVENTION: Tissue-specific poplar promoters

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER: EP.97.202.507.6
(B) FILING DATE: 13-AUG-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1994 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Populus trichocarpa

(ix) FEATURE:
(A) NAME/KEY: CAAT_signal
(B) LOCATION:1841..1848

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION:1871..1876

(ix) FEATURE:
(A) NAME/KEY: promoter
(B) LOCATION:1..1994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ACCCTCTATG TGCACGCCTG TCACCCCCGC GGTGCCTTAC CTGCTCCTGG GCTTGCAGGA 360

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GGCATTTTGC TTTGAGCCTC CGACCATTCC CCCCTGCTCT TTTCTTATTC ACGATAGAAG 540

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TTCTTGTTAA AATAAAGAAA AAAATTCAAT TGAAATACAC TTCCCAGCTT TCGAATGTTT 660

CTATTATATT TTGGAAAACA TGTGAAAAAT ATTTTATTAA TGTTCACTTG AAAAAATGGT 720

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AATTTAAAAC AAAAAAATAA AATAAAATAA TTTAAATTTT TTTAAATACT TTTAAAACAT 900

AAAAAAAAAA CAGAATAGAA TTTAGACATA AAAAGTGTTA TTCTCGTCGT TTAATAGCGG 960

GAGGTGGTAA GAAACATGCT AAGCATTCAC AGTTTTGGAT ATTGATTATC CATGTGTTAC 1020

CCTAAAAAAT GCCTTTTTCC ATTTCATGAA ATCCTCATTT AATAGCAATT CTTTAAAGGA 1080

GGAGACTAGA GACAGAGGTG GCTGCTGTCA ACCTAGTCGG TGAATTTAAA CTTCAACCGG 1140

CACATATATG CATACCAAGT ATACCCTTTA CATCTGCCCT AATTAAGACT GTAAAACGGA 1200

TTTGGATTTT TGCCGACAAA GGCTAGTTTG TGGAGAAAAA ACAACGAAGA TAAATTTTTA 1260

GATGACAAAG TCAACAATAG TTCGAGAGAT TCTTTAAAGG ACTCATCCGT TGACGGAGGT 1320

GGCCATATGC TACCAACTCT TGGACGTGGA GTCCCTTTGG TAATTTCACC TATCCCTCAC 1380

CCAATTTCTA TTAGCAGTTA GCACATGTAA TTTATGATTG GTGAGCCCAG CACAAATCTT 1440

TTCCAGTTAA ACACATATAT TAATTTATGA TTAATTATTT AATTCTCTCC ACTCTTAACA 1500

AATTAATCAT ACATGGCATA ACATTTTAGC TTTTGATCTC GAGAATCTCT ACCTAACCAT 1560

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AACCCCCTCC CGGTTGGTAG CCGGTCCAGC CTCCCCGCTA CTCCAATTCA AACCGGGCTC 1860

TCATTTCCAA TAAATACCAC CCGCCCTTTA CCATTTTCGA TCAGGTTAGG CATCACTACC 1920

ATCATCAACA AAAAAAAAAA AAAAATCCAA GGCCAAGAAA GAGATCGTAG TTTAATTAGA 1980

AGATATACAC AATA 1994

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1363 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pcpulus trichocarpa

(ix) FEATURE:
(A) NAME/KEY: promoter
(B) LOCATION:1..1363

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION:1243..1249

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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TCTAGAGAAC ACGGTTTCAA CCGCGTTTCC AAACATGATT TAAATATACA CGGTATATTT     60
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TATTATTTCA ATTTATTTGT AAGAAAAAAA ACCTTAAAAA ACAATTATTA CCAAAACATC    240
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CCTGTCATGG AGAAAGATTC CATGGTTGAA GACTGTATGA TAAGGAAAAA TGTCATGAAC    360
TCATGGTATA AGTAATTTCC TATCCAATAA CAGCAAGCTT GATGTTAGGT TAGGGTTGAT    420
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CGTGGAGTCA AATTCTTGAG GACACCTCAC CAACCCCTTA CCCACTTTCT ATTAGCAGCA    900
CATGTAGCCA TCCCCAACAA CAAAGTGGTG AGCCCACCAC AATTTTCTAC TCTCTACGAT    960
TTAAATCAAT TACACGTGGC ATAAAATGTC GAGCCTTTTA TTTCAAGAAA CCAAACCTAA   1020
CACCGTGAAC TTAATTTCTT TCGCAAATAT CTAAAATAAG GGTACATGAA TTAAATGTAT   1080
AGAAATTGAA TTAGTGTCCG AAACCTAAAA TGACCACTGG ACAAACACCG ATAAGTGGGT   1140
CCCAAAATAC CCACGGTGTC CTAAGAACTC ACCAACCCCC ACCCGGTTGG AAGCCGGTCC   1200
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AACCACCCCA CTACTCCGGC TCAAACCGGA CTCTCATCTC CAATAAATAC CACCTGCCCT 1260

TGCCATTTTC AATCAGGTCA GACATCCTTA CCATCGTCGC CCCCAGAAAA ACCTTCCAAC 1320

GCCAGGAAAG AGAGTATAGT TTTGTTATAA GATATACAAA ATA 1363

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: TISSUE-SPECIFIC POPLAR PROMOTERS

(57) Abstract

The invention concerns the isolation and characterization of DNA sequences representing a caffeoyl-CoA-O-methyltransferase (CCoAOMT) promoter having a biological activity in at least one plant or tree vessel and/or in cells adjacent to said vessel or vessels. The cells adjacent to the vessel are xylem ray cells whereas the vessel can be a differentiating vessel.

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INTERNATIONAL SEARCH REPORT

International Application No
PC[illegible] 98/04988

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHEN, C., ET AL.: "Populus trichocarpa CCoAOMT1 gene, exon 1 to exon 5" EMBL ACCESSION NO. AJ223621,17 July 1998, XP002086171 see the whole document ---	1
A	GRIMMIG, B., ET AL.: "Structure of the parsley caffeoyl-CoA O-methyltransferase gene, harbouring a novel elicitor responsive cis-acting element" PLANT MOLECULAR BIOLOGY, vol. 33, January 1997, pages 323-341, XP002054325 see the whole document ---	1-10
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 March 1999	19/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MENG, H., ET AL.: "Populus tremuloides caffeoyl-CoA 3-O-methyltransferase mRNA" EMBL SEQUENCE DATABASE, REL.44 13-JUN-1995, ACCESSION NO. U27116, XP002054772 see the whole document ---	1-10
A	YE, Z.-H., ET AL.: "Differential expression of two O-methyltransferases in lignin biosynthesis in Zinnia elegans" PLANT PHYSIOLOGY, vol. 108, 1995, pages 459-467, XP002054326 cited in the application see the whole document ---	1-10
A	CAPELLADES, M., ET AL.: "The maize caffeic acid O-methyltransferase gene promoter is active in transgenic tobacco and maize plant tissues" PLANT MOLECULAR BIOLOGY, vol. 31, 1996, pages 307-322, XP002054327 see page 314 - page 315 ---	1-10
A	FEUILLET, C., ET AL.: "Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants" PLANT MOLECULAR BIOLOGY, vol. 27, 1995, pages 651-667, XP002054328 cited in the application see the whole document ---	1-10
A	BEVAN, M., ET AL.: "Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants" EMBO JOURNAL, vol. 8, 1989, pages 1899-1906, XP002054329 cited in the application see the whole document ---	1-10
A	HAWKINS, S., ET AL.: "Cinnamyl alcohol dehydrogenase: identification of new sites of promoter activity in transgenic poplar" PLANT PHYSIOLOGY, vol. 113, February 1997, pages 321-325, XP002054330 cited in the application see the whole document ---	1-10

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAUFFE, K.D., ET AL.: "Combinatorial interactions between positive and negative cis-acting elements control spatial patterns of 4CL-1 expression in transgenic tobacco" THE PLANT JOURNAL, vol. 4, 1993, pages 235-253, XP002054331 cited in the application see the whole document ---	1-10
A	EP 0 516 958 A (BAYER AG) 9 December 1992 see page 4, line 1 - line 9 -----	1-5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/04988

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0516958 A	09-12-1992	DE 4117747 A	03-12-1992
		CA 2067317 A	01-12-1992
		JP 5199886 A	10-08-1993
		US 5728570 A	17-03-1998

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